REMARKS

In response to the Office Action dated January 10, 2001, Applicants have amended the claims which, when considered with the following remarks, is deemed to place the present application in condition for allowance. No new matter is introduced. Favorable consideration of all pending claims is respectfully requested.

Claims 6-12 and 41-51 are pending. In the pending Office Action, the Examiner alleges that claims 41-49 are drawn to a non-elected invention and are withdrawn from consideration.

Applicants respectfully traverse the Examiner's restriction in this regard. Applicants respectfully submit that an Examiner's authority to require restriction is defined and limited by statute:

> If two or more independent and distinct inventions are claimed in one application, the Commissioner may require the application to be restricted to one of the inventions.

35 U.S.C. § 121, first sentence (emphasis added). The implementing regulations of the Patent and Trademark Office include the mandate that restriction is appropriate only in cases presenting inventions which are both independent and distinct, 37 C.F.R. §§1.141-142. Without a showing of independence and distinctness, a restriction requirement is unauthorized.

Applicants respectfully submit that the subject matter of claims 41-49 is not "independent and distinct" from the subject matter of claims 6-12 and 50-51. More specifically, claim 6-12 and 50-51 are drawn to isolated nucleic acid molecules encoding a protein which comprises a SOCS box as defined by SEQ ID NO: 51. Claim 41 is also directed to an isolated nucleic acid molecule encoding a protein which comprises a SOCS box as defined by SEQ ID NO: 51 wherein X_1 is L, I, V, M or P (as opposed to L, I, V, M, A or P in claim 6). Claim 42,

depending from claim 6 and claim 41, further delineates the SOCS motifs of claims 6 and 41 by specific SEQ ID NOS. Claims 43-49 further delineate the nucleic acid molecules of claims 6 and 41 by sequence similarity or hybridization characteristics. Applicants respectfully submit that the subject matter of claims 6-12 and 50-51 and the subject matter of claims 41-49 are closely related, and not independent or distinct. Accordingly, Applicants respectfully request the Examiner reconsider the restriction and include all pending claims in the examination, or at least examine all pending claims (i.e., claims 6-12, 41-51) to the extent that these claims read on the originally elected species (SEQ ID NO: 3 and SEQ ID NO: 4).

In the pending Office Action, claims 6-12 and 50-51 are rejected under 35 U.S.C. §101. The Examiner alleges that the claimed nucleic acid molecules are not supported by either a specific and substantial asserted utility or by a well-established utility. More specifically, the Examiner contends that the binding partners and effector molecules of the SOCS proteins are unknown.

Applicants respectfully submit that a specific and substantial utility of the claimed nucleic acid molecules has been expressly asserted in the specification. For example, at page 61 of the specification, it is stated:

> The SOCS and its genetic sequence of the present invention will be useful in the generation of a range of therapeutic and diagnostic reagents and will be especially useful in the detection of a cytokine involved in a particular cellular response or a receptor for that cytokine.
>
> .....
>
> Soluble SOCS polypeptides are also contemplated to be particularly useful in the treatment of disease, injury or abnormality involving cytokine-mediated cellular responsiveness such as hyperimmunity, immunosuppression, allergies, hypertension and the like.

Furthermore, the specification provides that the claimed nucleic acid molecules can be used in a range of genetic based diagnostic assays screening for individuals with defective SOCS genes which lead to a range of conditions. See page 62 of the specification.

Applicants further submit that, even if the binding partners or the effector molecules of the SOCS proteins were unknown, such information is not entirely necessary for those skilled in the art to use the claimed nucleic acid molecules. In this regard, Applicants respectfully direct the Examiner's attention to Examples 13-14 of the specification (page 71) where the expression of the SOCS1 gene was shown to suppress the action of a range of cytokines including IL-6.

In this connection, Applicants respectfully submit that it was well-known in the art at the time the present application was filed that IL-6 was a multifunctional cytokine and that dysregulated overproduction of IL-6 causes unfavorable clinical symptoms in patients with immune-inflammatory diseases such as rheumatoid arthritis. See, e.g., Madhok et al. *Ann. Rheum. Dis.* 52 (3): 232-4 (1993) (the abstract of which is provided herewith as **Exhibit A**). Overproduction of IL-6 was also believed to be involved in the pathogenesis of lymphoid malignancies, e.g., high-grade B-cell lymphomas and myelomas. See, e.g., Emilie et al. *Blood* 80(2): 498-504 (1992) (the abstract of which is provided herewith as **Exhibit B**). Applicants further provide herewith three references showing the benefits of anti-IL-6 antibodies in the treatment of B-lymphoid malignancy and arthritis. See **Exhibits C-E**. Thus, in light of the present teaching, it would have been apparent to those skilled in the art that the claimed nucleic acid molecules can be used in the treatment of disorders wherein the suppression of the IL-6 function is desired.

As further support of the utility of the claimed nucleic acid molecules in the treatment of cytokine-mediated disorders, Applicants provide herewith two additional references:

Alexander et al. *Cell* 98: 597-608 (1999) (**Exhibit F**) which suggests that SOCS1 inhibits IFN-γ action; and Metcalf et al. Nature 405: 1069 (2000) (**Exhibit G**) which suggests that SOCS2 inhibits IGF-I action. Therefore, the claimed nucleic acid molecules can also be used in the treatment of disorders mediated by these cytokines.

In view of the foregoing, it is respectfully submitted that the claimed nucleic acid molecules are adequately supported by a substantial and specific utility as required by 35 U.S.C. §101. Withdrawal of the rejection under 35 U.S.C. §101 is therefore respectfully requested.

Claims 6-12 and 50-51 are further rejected under 35 U.S.C. §112, first paragraph. The Examiner contends that, since the claimed invention is not supported by either a specific and substantial utility or a well-established utility, one skilled in the art would not know how to use the claimed invention.

Applicants reassert that the claimed nucleic acid molecules are supported by a specific and substantial utility, and that the present specification adequately teaches one skilled in the art how to make and use the claimed nucleic acid molecules. As such, the rejection under 35 U.S.C. §112, first paragraph, is obviated. Withdrawal of the rejection is respectfully requested.

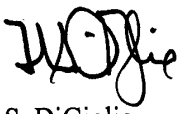
Claims 6-12 and 50-51 are further rejected under 35 U.S.C. §112, first paragraph as allegedly not enabled. The Examiner contends that it would take undue experimentation to make and use the molecules as claimed.

Applicants respectfully submit that the specification provides adequate guidance for those skilled in the art to make and use the SOCS molecules as claimed. In particular, the specification specifically exemplifies the isolation of multiple SOCS genes from more than one species, including murine SOCS-1, rat SOCS-1, human SOCS1,

murine SOCS-2, murine SOCS-3 and murine SOCS-5. The specification also provides partial or nearly full sequences for a number of other SOCS genes. In light of the present teaching, those skilled in the art can readily obtain a SOCS gene as claimed without undue experimentation. Further, as elucidated above, the use of such nucleic acids is also apparent from the teaching of the specification and the art extant at the time of the filing of the present application. As such, Applicants respectfully submit that the rejection under the enablement requirement of 35 U.S.C. §112, first paragraph, is overcome. Withdrawal of the rejection is respectfully requested.

In view of the foregoing amendments and remarks, it is firmly believed that the subject application is in condition for allowance, which action is earnestly solicited.

Respectfully submitted,



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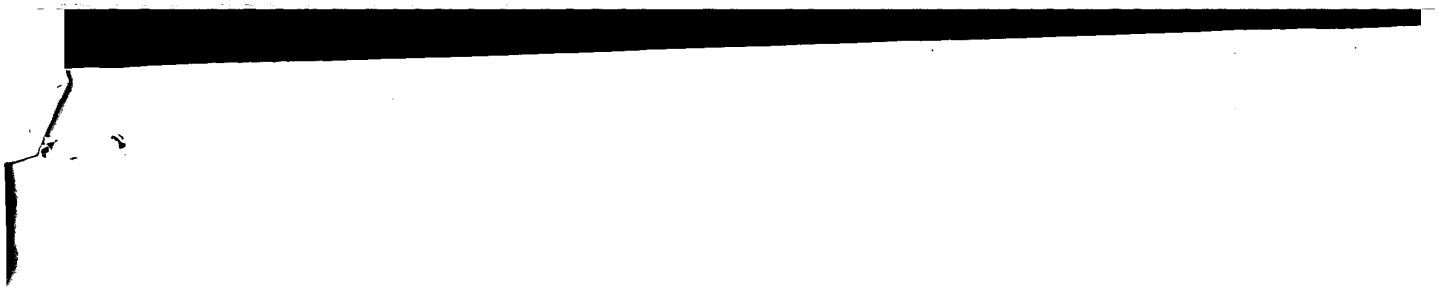
Encs.: Exhibits A-G



1: Ann Rheum Dis 1993 Mar;52(3):232-4 Related Articles, Books, LinkOut

Serum interleukin 6 levels in rheumatoid arthritis: correlations with clinical and laboratory indices of disease activity.

Madhok R, Crilly A, Watson J, Capell HA.

Department of Rheumatology, Gartnavel and Western Infirmary, Glasgow, United Kingdom.

In rheumatoid synovium interleukin 6 (IL-6) is the most abundantly expressed cytokine. Increased serum levels have been previously reported in patients with rheumatoid arthritis (RA). In this study serum IL-6 levels were measured in a well defined cohort using a bioassay (B9 cells) and levels were correlated with conventional clinical and laboratory indices of disease activity. Levels were significantly higher in serum from patients with RA (median 55 IU/ml; interquartile range 28-139) compared with serum from disease (median 7 IU/ml; 1-23) and normal controls (median 10 IU/ml; 7-12). No difference was observed between men and women. Levels did not correlate with disease duration. Significant associations were observed between IL-6 and C reactive protein and between the Ritchie articular index and duration of morning stiffness. No other correlations were observed. The value of these findings in the monitoring of RA and as an indicator of response to second line treatment needs to be established.

PMID: 8484679 [PubMed - indexed for MEDLINE]

1: Blood 1992 Jul 15;80(2):498-504 Related Articles, Books, LinkOut

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Interleukin-6 production in high-grade B lymphomas: correlation with the presence of malignant immunoblasts in acquired immunodeficiency syndrome and in human immunodeficiency virus-seronegative patients.

Emilie D, Coumbaras J, Raphael M, Devergne O, Delecluse HJ, Gisselbrecht C, Michiels JF, Van Damme J, Taga T, Kishimoto T, et al.

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The mechanisms leading to malignant cell proliferation may differ between the different histologic forms of high-grade non-Hodgkin's lymphomas. To analyze the potential role of interleukin-6 (IL-6) as a growth factor for lymphomatous cells in these different forms, the in situ production of this cytokine was analyzed in lymphomatous samples taken from 24 patients, 18 of whom were human immunodeficiency virus (HIV) infected. Eleven Burkitt's lymphomas (BLs), seven diffuse large-cell lymphomas, and six immunoblastic lymphomas were studied. In situ hybridization experiments showed that the IL-6 gene was expressed in all tissues. The number of IL-6 gene-expressing cells was 7 times higher in the non-BLs than in the BLs, and it was 17 times higher than that of 14 control lymph nodes displaying a benign follicular hyperplasia. Analysis of individual cases indicated that the level of IL-6 gene expression was strongly correlated with the presence of immunoblasts within the malignant clone. In contrast, this level was not correlated with the presence of Epstein-Barr virus genome in the lymphoma or with the HIV status of patients. Immunohistochemical studies with an anti-IL-6 monoclonal antibody showed that IL-6 was produced in non-BLs, but not in BLs. In the former, IL-6 mainly originated from reactive, nonmalignant cells. Immunohistochemical analyses of non-BLs also showed that malignant cells produced the 80-Kd chain of the IL-6 receptor. Taken together, these results suggest that IL-6 may act as a growth factor in some forms of high-grade B lymphomas. The presence of immunoblasts may be an indicator of such forms.

PMID: 1320956 [PubMed - indexed for MEDLINE]

Treatment of B-lymphoproliferative disorder with a monoclonal anti–interleukin-6 antibody in 12 patients: a multicenter phase 1-2 clinical trial

Elie Haddad, Sophie Paczesny, Veronique Leblond, Jean-Marie Seigneurin, Marc Stern, Antoine Achkar, Marc Bauwens, Vincent Delwail, Dominique Debray, Christophe Duvoux, Philippe Hubert, Bruno Hurault de Ligny, John Wijdenes, Anne Durandy, and Alain Fischer

Severe T-cell immunodeficiency after solid organ or bone marrow transplantation may result in the uncontrolled outgrowth of latently Epstein-Barr virus–infected B cells, leading to B-lymphoproliferative disorder (BLPD). Given the potentially important pathogenic role of IL-6 in BLPD, it was tested whether the in vivo neutralization of IL-6 by a monoclonal anti–IL-6 antibody could contribute to the control of BLPD. Safety and efficacy were assessed in 12 recipients of transplanted organs who had BLPD refractory to the reduction of immunosuppression over 8 days. Five patients received 0.4 mg/kg per day. The next 7 patients received 0.8 mg/kg per day. Treatment was scheduled to last 15 days. It was completed in 10 patients, and in the other 2 patients was discontinued early (days 10 and 13, respectively) because of disease progression. Treatment tolerance was good, and no major side effects were observed. High C-reactive protein levels were found in 9 patients before treatment but were normalized under treatment in all patients, demonstrating efficient IL-6 neutralization. Complete remission (CR) was observed in 5 patients and partial remission (PR) in 3 patients. Relapse was observed in 1 of these 8 patients in whom remission was observed. This relapse was unresponsive to treatment. Disease was stable in 1 patient, but it progressed in 3 patients. Seven patients are alive and well. Two patients died because of disease progression, and 3 patients died while in CR (chronic rejection in 2 patients and BLPD sequelae in 1 patient). These data suggest that the anti–IL-6 antibody is safe and should be further explored in the treatment of BLPD. (Blood. 2001;97: 1590-1597)

Introduction

B-lymphoproliferative disorder (BLPD) is a severe complication of organ and bone marrow transplantation (BMT) caused by the Epstein-Barr virus (EBV).[1-7] EBV latently infects B cells, which become immortalized by expressing part of the viral genome that persists in an episomal form.[8] The growth of EBV-infected B cells is controlled by cytotoxic T cells.[9] In vivo severe T-cell immunodeficiency, such as occurs in patients with inherited cellular immunodeficiency[10] or acquired immunodeficiency syndrome[11] and in recipients of solid organ or bone marrow transplants,[12-14] may result in the uncontrolled outgrowth of EBV-infected B cells, leading to BLPD. BLPD occurs in 1% to 15% of organ recipients and in 0.5% to 24% of bone marrow recipients, depending on the type of transplantation, the ages of donor and recipient, the intensity of immunosuppression, and the method of T-cell depletion.[15-18] The overall prognosis of BLPD is poor, and the disease is fatal in 40% to 60% of affected recipients of transplanted organs[15,19-22] and in 90% of affected BMT recipients.[15,23-25] Treatment strategy is still controversial. Antiviral therapy (acyclovir, ganciclovir) is ineffective at preventing the persistence of episomal EBV associated with the latent phase. Although such treatment has not been definitively shown to be effective against BLPD, remission has occasionally been reported.[15,26] Chemotherapy and radiotherapy are of limited value, at least in early-onset EBV-associated BLPD.[15,27] Surgery may save the patient's life in cases of localized BLPD.[15] Preliminary data have suggested improved survival with the use of interferon-α and the intravenous infusion of high-dose immunoglobulins.[28] For BLPD occurring after BMT, the infusion of donor T lymphocytes or EBV-specific donor cytotoxic T lymphocytes can be effective in bringing B-cell proliferation under control.[12,29] The use of anti–B-cell monoclonal antibodies (mAbs) (anti-CD24 and anti-CD21) appeared to be a safe and relatively efficient therapy for post-transplant early-onset BLPD.[30-32] However, these anti–B-cell mAbs are no longer available. The use of a humanized anti-CD20 mAb may be an attractive alternative, as recently reported in a small number of patients.[33-35]

We investigated the effect of a monoclonal anti–interleukin-6 (IL-6) antibody on B-cell growth in patients with BLPD. IL-6 is a multifunctional cytokine produced by monocytes, fibroblasts, endothelial cells, and other cell types. It plays an important role in the proliferation and maturation of B cells.[36-39] Overproduction of this cytokine is thought to be involved in the pathogenesis of lymphoid malignancies, high-grade B-cell lymphomas,[40,41] and myelomas[42-44] in particular. It has also been shown that IL-6 promotes the growth of EBV-infected B cells,[45,46] that patients with BLPD produce abnormally high levels of IL-6,[45,47] and that B-cell lines derived from BLPD express the p80 chain of the IL-6 receptor.[48] In

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addition, the transfection of EBV-transformed B cells with human IL-6 cDNA greatly increases the proliferation of these cells both in vivo and in vitro.[49,50] Durandy et al[48] showed that anti–IL-6 mAb could inhibit the growth of several BLPD-derived B-cell lines in severe combined immunodeficiency disease (SCID) mice in vivo. This treatment led to complete remission in most mice and to tumor-free, long-term survival in 40% of mice.

Given the probable importance of IL-6 in BLPD pathogenesis, we tested, in a phase 1-2 clinical trial, the toxicity and efficacy of anti–IL-6 mAb treatment against BLPD occurring after organ transplantation. We report here the results of a trial in 12 patients.

Materials and methods

Monoclonal anti–IL-6 antibody

The mouse monoclonal anti–IL-6 antibody (B-E8)[51] was produced and supplied by Diaclone (Besançon, France; specific activity, 1 μg B-E8 neutralizes 6000 U IL-6).

Protocol design

This study consisted of a multicenter phase 1-2 trial of monoclonal anti–IL-6 antibody administration to patients with post-transplant BLPD. Detailed informed consent was obtained from all patients or from parents of children younger than 10 years of age, in accordance with French legislation and as approved by the ethics committee of Necker Hospital (Paris, France). We evaluated anti–IL-6 antibody-related toxicity and effects on BLPD.

Patient enrollment

BLPD was diagnosed based on the presence of a lymphoproliferative syndrome with detectable tumors consisting of EBV-positive B lymphocytes. EBV was detected immunohistologically, with anti-LMP antibodies, by polymerase chain reaction (PCR) detection of the EBV genome, or by Southern blot analysis or by in situ hybridization (see below).

For inclusion in the study, patients could be of any age but had to have acquired BLPD after organ transplantation and to have satisfied at least one of the following criteria: (1) be unresponsive to the tapering of immunosuppression for a minimum of 8 days; (2) have histologically invasive disease with nodal capsule disruption; (3) have rapidly progressive multiple lymphoproliferative lesions, excluding those from surgery. BLPD unresponsive to the tapering of immunosuppression treatment for a minimum of 8 days was defined as no change in C-reactive protein (CRP) level, fever, or other general manifestations, and no decrease in tumor size detected by clinical assessment and appropriate imaging tests.

No other BLPD treatments were given in association with the anti–IL-6 antibody. If disease progression was observed during the administration of anti–IL-6 antibody, treatment could be stopped and another treatment could be proposed. Twelve patients were included in the study between September 1995 and August 1998. The final data were collected on June 30, 1999.

Anti–IL-6 antibody dose and administration

The monoclonal anti–IL-6 antibody BE-8 was diluted in 2 mL/kg 5% glucose serum and administered as a 30-minute intravenous infusion once a day for 15 days. To evaluate toxicity and the dose dependency of the effects of the anti–IL-6 antibody, a dose escalation trial was performed. Patients 1 to 3 received a dose of 0.4 mg/kg per day. Patients 4 and 5 received a 1 mg/kg bolus on day 1, which was followed by injections of 0.4 mg/kg per day. These doses were well tolerated (see "Results"); hence, the remaining 7 patients received a third regimen consisting of a 1 mg/kg bolus followed by injections of 0.8 mg/kg per day. CRP was determined on day 3. In all patients, if CRP levels did not normalize by day 3, the dose given was doubled for the remaining 12 days of treatment, whatever the initial dose.

Study criteria

The end point of this dose escalation study was the determination of the toxicity of anti–IL-6 antibody administration and its efficacy against BLPD, as assessed 4 months after treatment. We evaluated the toxicity of BE-8 in patients using the World Heath Organization Toxicity Criteria. Blood pressure, temperature, and heart rate were monitored every 10 minutes during BE-8 infusion, then every hour for 3 hours and every 3 hours until the next injection. Hematologic, renal, and liver function tests were conducted every day during treatment and on days 22, 30, 60, and 120.

Clinical symptoms of BLPD were monitored (examination of all affected sites, fever) every day during treatment, then once per week for 2 weeks, and then once per month for 6 months. Tumor size was determined by radiologic imaging (computed tomography and/or magnetic resonance imaging and/or ultrasound imaging) on days 15, 30, and 120. Biologic variables were also studied. CRP was determined every 3 days during treatment, then once per week for 2 weeks, and then on days 60 and 120. PCR tests for the detection of EBV-DNA in blood and determinations of serum immunoglobulin levels, monoclonal immunoglobulin components, and specific antibodies against EBV were carried out every 2 weeks for 1 month and then on days 60 and 120.

Complete remission (CR) was defined as the complete clinical and radiologic disappearance of tumors at all sites, the disappearance of associated biologic signs (high levels of CRP, detection of circulating B cells expressing the EBV genome), and the absence of newly involved sites. Partial remission (PR) was defined as at least a 50% decrease in measurable tumor localization, with the disappearance of fever and no newly involved sites. Stable disease (SD) was defined as no significant change in tumor measurements and no newly involved sites. Progressive disease (PD) was defined as an increase in the size of tumor lesions or the appearance of new lesions.

Immunologic investigations

The following B- and T-cell–specific mAbs were used, as previously described,[31] to characterize T and B lymphocytes in blood and in organ tissue samples when available: anti-immunoglobulin heavy-chain and light-chain isotypes[30]; anti-CD19, CD20, CD24, CD21, and CD23 antibodies (Immunotech, Marseilles, France); and anti-CD3, CD4, and CD8 antibodies (BD, San Diego, CA). Analyses were performed by indirect immunofluorescence cytofluorometry. Fresh cells were used for membrane immunofluorescence analysis and fixed cells for intracytoplasmic staining. Immunoperoxidase staining of biopsy sections was performed as previously described.[52] Serum immunoglobulin levels were determined by nephelometry, and monoclonal immunoglobulin components were determined by immunofixation.[53] Serum IL-6 levels were determined using a specific anti–IL-6 enzyme-linked immunosorbent assay (ELISA) assay (CLB, Amsterdam, The Netherlands), as previously described.[48]

Virologic investigations

EBV-DNA was detected in frozen material by Southern blot analysis using a randomly primed ^{32}P-labeled probe specific for the *Bam*HI W internal repeats of the virus, in situ hybridization with EBV-specific probes,[54] PCR analysis,[55] or any combination thereof. Specific antibodies (IgG and IgM isotypes) against EBV (viral capsid antigen, early antigen, EBV nuclear antigen) in organ transplant recipients were detected by an ELISA assay. Immunoperoxidase staining of biopsy sections was also performed for LMP1.[52]

Cl nality studies

Immunoglobulin gene rearrangement studies of proliferative B cells were performed by Southern blotting, using a probe for the sequence encoding the heavy-chain joining region (JH). BLPD was considered to be monoclonal if a single immunoglobulin rearrangement was obtained for the abnormal specimen analyzed, regardless of whether a single monoclonal serum component had been detected by immunofixation. BLPD was considered to be oligoclonal if no unique immunoglobulin heavy-chain

rearrangement was observed, several serum monoclonal immunoglobulin components were detected through immunofixation, or both.

Results

Patients and BLPD characteristics

Median age at the onset of BLPD was 35 years (range, 1 year to 62 years). Organs transplanted were lung (n = 5), kidney (n = 3), liver (n = 3), and heart (n = 1). The characteristics of the BLPD are shown in Table 1. In all patients, biopsy of BLPD lesions was performed to confirm the B-lymphocyte phenotype of infiltrating cells and the presence of EBV (Table 1).

All patients were on immunosuppressive treatment at the time of BLPD onset (Table 2). Eight patients had received highly aggressive immunosuppressive therapy because of graft rejection; in 4 patients, this occurred less than 3 months before the onset of BLPD. In all patients, immunosuppression was reduced when BLPD was diagnosed. In none of these patients did immunosuppression tapering for at least 8 days affect the BLPD lesions (ie, tumors were not reduced, as shown by appropriate imaging and clinical examination). Tapering of immunosuppression also had no effect on fever or serum CRP levels. All patients were, therefore, considered unresponsive to the tapering of immunosuppression over an 8-day period.

One patient (patient 12) had been injected with an anti-CD20 antibody that had no effect on BLPD, 2 months before anti–IL-6 antibody treatment. In all other patients, no other treatment was performed before the initiation of anti–IL-6 mAb antibody therapy.

Treatment characteristics and tolerance

Treatment was scheduled to last 15 days. It was completed in 10 patients, and in the other 2 patients it was stopped early (on day 10 for patient 4 and on day 13 for patient 7) because of disease progression. One patient (patient 2) received a second course of anti–IL-6 antibody treatment after a relapse of BLPD. No major side effect was observed during any of the 13 courses of anti–IL-6 antibody treatment. One patient (patient 4) had moderate allergic manifestations—erythema on both hands after each anti–IL-6 antibody injection—that responded to antihistamine treatment. One patient (patient 3) had paresthesia on day 2, which then disappeared spontaneously. Two patients (patients 2, 9) had moderately high levels of arterial blood pressure, which resolved after sublingual nifedipine treatment. On day 2, patient 3 was examined for hyperkalemia associated with hyperphosphoremia and hypocalcemia, all of which resolved within 1 day. These biochemical manifestations were interpreted as a lytic syndrome.

Pharmacologic data

As high CRP concentration is known to be an in vivo hallmark of high levels of IL-6 synthesis.[56-58] We assessed the effects of the treatment on CRP and IL-6 levels to determine the pharmacologic effects of the anti–IL-6 antibody. High serum CRP concentrations were found in 9 of 12 patients, and 5 of 9 patients had fever (Table 1). In all patients, CRP concentration decreased and fever disappeared after treatment, suggesting that systemic IL-6 was neutralized (Figure 1). CRP normalized within 3 days of the initiation of treatment in 7 of these 9 patients, whereas it was necessary to double the anti–IL-6 antibody dose on day 3 for 2 patients (patients 6, 11) even though the initial dose given to these patients was 0.8 mg/kg.

As shown in Table 3, serum IL-6 concentration was evaluated in 8 patients before treatment (after tapering immunosuppression) and was found to be elevated in 5 patients. In the 4 patients who survived for more than 2 months, IL-6 normalized or decreased significantly. Serum IL-6 concentration could not be analyzed during or immediately after treatment because of the formation of immune complexes between IL-6 and anti–IL-6 antibody that were also detected in the ELISA assay.

Evaluation of anti–IL-6 antibody treatment on disease outcome

Twelve patients were treated with 13 courses of anti–IL-6 antibody injection. Median follow-up time after treatment was 20 months (range, 9-33 months). CR was achieved in 5 patients (patients 1, 3, 5, 6, 8), 4 months after the initiation of treatment (Table 3). In patient 1, tumor size had decreased by 50% on day 12 of treatment, and CR was achieved on day 21. Examination of an endobronchial lesion on day 21 showed that no infiltrating tumor cells were present. Neither necrosis nor T-cell infiltration was detected. Patient 3 presented on day 2 with hyperkalemia associated with hyperphosphoremia and hypocalcemia, suggestive of a lytic syndrome. By day 3, the tumor size had decreased, and CR was achieved 5 weeks after the initiation of treatment. It was not possible to perform an examination of the lesion after CR was achieved. In patient 5, tumor size began to decrease 30 days after treatment, and CR was achieved 4 months after treatment. Examination of the lungs confirmed that infiltrating tumor cells were no longer present and that there was no necrosis, nor were there infiltrating T cells. In patient 6, tumor reduction was observed 30 days after treatment, and CR was achieved 2 months after treatment. Analysis showed that the infiltrating cells had been replaced by severe necrosis. In patient 8, pleuritis had disappeared by day 3, and CR was achieved on day 15. No pathologic examination was performed.

None of these patients had a relapse of BLPD. All can be considered cured of BLPD. However, 3 of these 5 patients died 10, 13, and 36 months after treatment because of chronic rejection in 2 patients and severe liver hilum necrosis in the other, which may be considered a sequela of BLPD. Two patients are alive and well 21 and 33 months, respectively, after anti–IL-6 antibody treatment.

Partial remission was observed in 3 patients (patients 2, 9, 12). In patient 2, pleurisy decreased by the third day after treatment, mediastinal nodes were half their size 1 month after treatment, and BLPD decreased by approximately 90% only 2 months after treatment. However, BLPD relapsed 5 months after treatment. This patient received a second course of anti–IL-6 antibody treatment, which was ineffective. This patient eventually died of progressive disease. In patient 9, a decrease in tumor size became detectable 15 days after the initiation of treatment. This PR made it possible to perform surgery and retransplantation that had not previously been feasible. Pathologic examination of the liver showed necrosis of 80% of the tumor that was infiltrated by T cells and histiocytic cells. This patient was alive and well, in complete remission, at the 21-month follow-up. In patient 12, a slight decrease in tumor size was observed by day 30. Three months later, the tumor decreased by 90%. Nine months after treatment, this patient was still in PR and had a small persistent lesion in the lung but no associated manifestations. Before the anti–IL-6 antibody treatment, this patient received a course of anti-CD20 antibody that had no effect on BLPD.

Anti–IL-6 antibody treatment was effective in 8 patients (5 in

Table 1. Patients and BLPD characteristics

Patient/sex	Age (y)	Disease	Transplant	Affected sites	General manifestations	Pathologic morphology of BLPD lesions	Phenotype of infiltrating cells in BLPD lesions	Clonality of BLPD lesions	EBV in BLPD lesions	Detection of EBV genome by PCR out of tumor site	Circulating B cells (CD19 or CD20/μl)
1/F	41	Lymphoangiomatosis	Unipulmonary	Endobronchial	None	Polymorphic necrosis	$CD20^+$	Polyclonal	$LMP1^+$ $EBER^+$	ND	80
2/M	61	OLD	Bipulmonary	Bone, bone marrow, pleura, mediastinal nodes	Fever, high CRP level	Monomorphic, plasmacytoid	IgA_κ^+	Polyclonal	$EBER^+$	ND	ND*
3/M	33	Renal artery trauma	Kidney	Lymph nodes, liver	Fever, high CRP level	Polymorphic	$CD20^+$ $CD79^+$ $CD30^+$	ND	$LMP1^+$	Blood	6
4/F	27	Pulmonary hypertension	Bipulmonary	Oral	None	Monomorphic necrosis	$CD30^+$	Monoclonal	$LMP1^+$ $EBER^+$	ND	20
5/M	38	Cystic fibrosis	Bipulmonary	Lungs	None	Polymorphic necrosis	$CD20^+$ $CD79^+$	ND	$LMP1^+$ $EBER^+$	Blood bone marrow CSF	92
6/M	62	Liver cirrhosis	Liver	Liver hilum	High CRP level	Polymorphic necrosis	$CD20^+$ $CD79^+$	Polyclonal	$LMP1^+$	Blood	14
7/M	49	NOCM	Heart	Perinephritis, cutaneous	High CRP level	Monomorphic plasmacytoid	κ $chain^+$	Monoclonal	$Southern^-$ PCR^-	ND	ND*
8/M	1	Biliary atresia	Liver	Liver, spleen, lymph nodes pleura	Fever, high CRP level	Polymorphic	$CD20^+$	Monoclonal	PCR^+	Blood, CSF,	40
9/M	12	α_1Anti-trypsine deficiency	Liver	Liver hilum, graft (liver), bone marrow	Fever, high CRP level	Polymorphic necrosis	$CD19^+$ $CD20^+$ $CD22^+$	Monoclonal	$EBER^+$ PCR^+	Pleursy blood, bone marrow	45
10/M	31	Interstitial nephritis	Kidney	Liver, abdomen, lymph nodes, subcutaneous	High CRP level	Polymorphic	$CD20^+$ $CD79^+$	ND	$LMP1^-$	Blood	ND
11/M	32	Kidney hypoplasia	Kidney	Graft (kidney), kidney hilum, lymph nodes	Fever, high CRP level	Polymorphic	$CD20^+$	ND	$LMP1^+$ $EBER^+$	ND	7
12/M	20	Cystic fibrosis	Bipulmonary	Lungs, mediastinal nodes	High CRP level	Polymorphic necrosis	$CD19^+$ $CD20^+$ $CD79^+$	Monoclonal	$LMP1^+$ $EBER^+$	Blood	None

OLD, obstructive lung disease; NOCM, nonobstructive cardiomyopathy; CSF, cerebrospinal fluid.
*In 2 patients, hyperbasophilic cells could be detected in blood.

Table 2. Immunosuppression tapering

Patient	Treatment of last rejection episode	Time from last rejection episode to BLPD onset (mo)	IS at BLPD onset	Reduction of IS	T-cell count before anti–IL-6 (CD3/μL)	Anti-EBV antibodies before treatment	Anti-EBV antibodies after treatment (day 30)
1	3 Pulse steroids	3	Steroids, CsA, azathioprine	Increase 33% steroids	660	IgM VCA$^-$	IgM VCA$^-$
				Stop azathioprine		IgG VCA$^+$	IgG VCA$^+$
				Reduction 50% CsA		IgG EBNA$^-$	IgG EBNA$^-$
2	Pulse steroids, ATG, OKT3	10	Steroids, CsA	Reduction 50% CsA	ND	IgM VCA$^-$	IgM VCA$^-$
						IgG VCA$^+$	IgG VCA$^+$
						IgG EBNA$^+$	IgG EBNA^{++}
3	Pulse steroids	103	Steroids, CsA, azathioprine	Stop steroids	ND	IgM VCA$^-$	IgM VCA$^-$
				Stop azathioprine		IgG VCA$^+$	IgG VCA$^+$
						IgG EBNA$^-$	IgG EBNA$^-$
						IgG EA$^+$	IgG EA$^-$
4	None	—	Steroids, CsA, azathioprine	Stop azathioprine	227	IgM VCA$^+$	ND
				Reduction 50% CsA		IgG VCA^{+++}	
						IgG EBNA$^-$	
5	3 Pulse steroids	1.5	Steroids, CsA, azathioprine	Stop CsA	1 074	IgG VCA$^-$	IgG VCA$^-$
				Stop azathioprine			
6	None	—	Steroids, FK506	Stop FK506	309	IgM VCA$^-$	IgM VCA$^-$
						IgG VCA$^+$	IgG VCA$^+$
						IgG EBNA$^+$	IgG EBNA$^+$
7	ATG	11	Steroids, CsA	Reduction 50% steroids	ND	IgM VCA$^-$	IgM VCA$^-$
				Reduction 50% CsA		IgG VCA$^+$	IgG VCA$^+$
						IgG EBNA$^+$	IgG EBNA$^+$
8	Pulse steroids	2.5	Steroids, FK506, azathioprine	Stop azathioprine	187	IgM VCA$^-$	IgM VCA$^-$
				Stop FK506		IgG VCA$^+$	IgG VCA^{++}
						IgG EBNA$^+$	IgG EBNA^{++}
9	None	—	Steroids, CsA	Stop CsA	322	IgM VCA$^-$	IgM VCA$^-$
						IgG VCA$^+$	IgG VCA^{++}
						IgG EBNA^{+++}	IgG EBNA^{+++}
						IgG EA^{++}	IgG EA$^+$
10	Pulse steroids	48	Steroids, CsA, azathioprine	Stop CsA	ND	IgM VCA$^-$	ND
				Stop azathioprine		IgG VCA$^+$	
				Reduction 50% steroids		IgG EBNA$^+$	
						IgG EA^{++}	
11	None	—	Steroids, CsA, mycophenolate	Stop CsA	ND	IgM VCA$^+$	IgM VCA^{+++}
				Reduction 50% mycophenolate		IgG VCA$^+$	IgG VCA^{+++}
						IgG EBNA$^+$	IgG EBNA^{+++}
12	Pulse steroids	0.5	Steroids, CsA, azathioprine	Stop CsA	840	IgM VCA$^-$	IgM VCA$^-$
				Stop azathioprine		IgG VCA$^+$	IgG VCA$^-$
						IgG EBNA$^-$	IgG EBNA$^-$
						IgG EA$^+$	IgG EA:ND

IS, immunosuppression; CsA, cyclosporin A; ATG, antithymoglobulin.

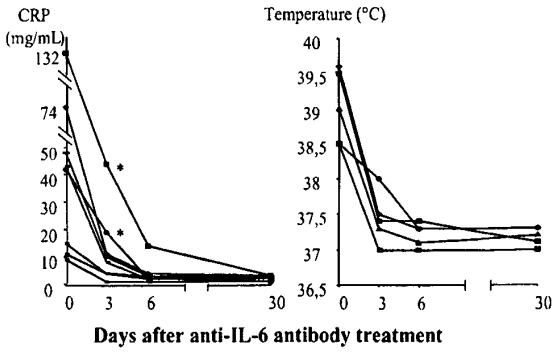

Figure 1. Evolution of CRP levels and fevers after anti–IL-6 antibody treatment. Asterisks indicate patients in whom the absence of CRP level normalization on day 3 led to a 2-fold increase in the anti–IL-6 treatment dose.

CR, 3 in PR). The reintroduction of highly aggressive immunosuppression in 3 of these patients because of episodes of rejection did not lead to BLPD relapse.

Stable disease was observed in one patient (patient 11), who was then treated with anti-CD20 antibody, which also failed to control BLPD. This patient was then treated by surgery and retransplantation and is alive and well, in complete remission, 19 months after treatment.

In 3 patients, treatment did not prevent disease progression (patients 4, 7, 10). Patient 10 died within 1 month because of disease progression, despite the use of chemotherapy. In patients 7 and 10, anti–IL-6 antibody treatment was stopped on days 10 and day 13, respectively, because of disease progression, but chemotherapy led to complete remission. These 2 patients are still alive and well and in CR 22 and 26 months, respectively, after treatment.

Anti–IL-6 antibody treatment was effective at controlling associated signs of BLPD such as high CRP concentration and

Table 3. Effects of anti–IL-6 antibody treatment on BLPD

Patient	Time from transplantation to BLPD onset (mo)	Anti–IL-6 treatment/bolus (mg/kg)	Serum IL-6 level (at day 0) (pg/mL)	Serum IL-6 level 60 days after treatment (pg/mL)	Effect on BLPD 4 mo after treatment	General outcome	Follow-up (mo)
1	6	No/0.4	< 1	3	CR	Died (36 mo) of chronic rejection	—
2-a*	84	No/0.4	< 1	< 1	PR	Relapse	—
2-b	90	No/0.4	< 1	—	PD	Died (7 mo) of BLPD	—
3	108	No/0.4	ND	ND	CR	A&W, CR	33
4	60	Yes/0.4	ND	ND	PD	Anti–IL-6 antibody treatment failure A&W, CR after chemotherapy	26
5	2	Yes/0.4	9	< 1	CR	Died (10 mo) of chronic rejection	—
6	6	Yes/0.8	13	< 1	CR	Died (13 mo) of liver hilum necrosis	—
7	156	Yes/0.8	< 1	ND	PD	Failed anti–IL-6 antibody treatment, A&W, CR after chemotherapy	22
8	3.5	Yes/0.8	58	15	CR	A&W, CR	21
9	3.5	Yes/0.8	46	< 1	PR	A&W, CR after surgery and retransplantation	21
10	84	Yes/0.8	15	—	PD	Died (1 mo) of BLPD	—
11	5	Yes/0.8	ND	ND	SD	Anti–IL-6 antibody treatment failure, A&W, CR after surgery	19
12	1	Yes/0.8	ND	ND	PR	A&W, PR	9

The sensitivity of ELISA test for IL-6 is 0.6 pg/mL. Levels indicated as < 1 pg/mL mean that no IL-6 could be detected by this ELISA test. When measured by this method, IL-6 levels in healthy controls were < 1 pg/mL.

ND indicates not done; A&W, alive and well.

*2-a and 2-b indicate the first and second course of anti–IL-6 antibody.

fever. This treatment was also shown to be effective by PCR detection of the EBV genome in the blood and the determination of serum immunoglobulin concentration by immunofixation. For 7 patients, the EBV genome was detected in the blood before treatment. We were able to evaluate 6 of these patients after treatment. The EBV genome was no longer detectable in the blood 2 to 4 months after treatment in 5 of these 6 patients. Six of 10 patients with detectable serum monoclonal immunoglobulin components on immunofixation were evaluated after treatment. In 5 of them, the monoclonal immunoglobulin components had disappeared. Most of the survivors developed full antibody responses to EBV, including anti-EBNA antibodies (Table 2).

Analysis of factors that might have influenced disease outcome

BLPD characteristics such as clonality, EBV genome detection in blood, monoclonal immunoglobulin component, number or localization of affected sites, and time to BLPD onset and patient characteristics such as age, type of transplantation, and type of initial disease were tested as possible factors influencing outcome after anti–IL-6 antibody treatment.

The dose of anti–IL-6 antibody given (0.4 vs 0.8 mg/kg) did not affect its efficacy (Table 3). Although no firm conclusions can be drawn because of the small number of patients, time to BLPD onset seemed to be the only factor affecting treatment efficacy. Indeed, for the 7 patients in whom BLPD occurred within 6 months of transplantation, CR was achieved in 4 patients and PR was achieved in 2 patients (Table 4). Conversely, for the 5 patients in whom BLPD occurred more than 5 years after BLPD, CR was achieved in only one patient and PR in another, who eventually had a relapse, whereas the disease progressed in 3.

Discussion

In this phase 1-2 clinical trial, 12 patients with BLPD after organ transplantation were treated intravenously by daily injections of 0.4 to 0.8 mg/kg per day of a mouse monoclonal anti–IL-6 antibody (B-E8)[51] for 15 days in 10 patients and for 10 and 13 days, respectively, in 2 other patients. This antibody has previously been used to treat patients with myeloma,[56] B lymphoma related to EBV infection in the patients with HIV,[59] Castleman disease,[60] and severe rheumatoid arthritis.[61] In all these studies, treatment was

Table 4. Influence of time to BLPD onset on anti–IL-6 antibody treatment efficacy

Time to BLPD onset	n	CR	PR	SD	PD
< 6 mo	7	4	2	1	0
> 5 y	5	1	1*	0	3

*This patient had a relapse; a second cure had no effect (PD).

well tolerated. The treatment was also well tolerated in this study, and no major side effects were reported for any of the doses of anti–IL-6 mAb used. These data suggest that this mAb is safe for use in therapeutic trials at doses up to 0.8 mg/kg per day for 15 days.

To determine the pharmacologic kinetics of anti–IL-6 mAb antibody, we analyzed CRP and IL-6 levels. CRP is produced by human hepatocytes in response to IL-6 stimulation[58] and is known to be indicative of the presence of high levels of IL-6.[56-58] Nine patients had high CRP levels. The normalization of serum CRP concentration by treatment in all 9 patients suggests that IL-6 was systematically neutralized. Two of these 9 patients, both of whom received an initial anti–IL-6 antibody dose of 0.8 mg/kg, had persistently high CRP levels after 3 days of treatment. The dose of anti–IL-6 mAb was therefore increased, resulting in the normalization of CRP concentration. This suggests that CRP determination during treatment may be useful for assessing whether a pharmacologic effect has been achieved. However, CRP concentration was not predictive of treatment efficacy.

Treatment was clinically effective in 8 patients (5 in CR, 3 in PR). BLPD relapsed in only one of these 8 patients and then became insensitive to treatment. These data indicate that the anti–IL-6 mAb may be useful in the treatment of severe BLPD.

In all cases, prior immunosuppression tapering for an 8-day period had no effect on BLPD. However, one could argue that this delay is too short and that the observed remissions could be related to a delayed effect of immunosuppression reduction. This is unlikely because the observed remission rate under immunosuppression tapering is generally lower than the one observed in our series (8 of 12 patients). Moreover, this 8-day period of immunosuppression reduction has been previously proposed as an appropriate delay to allow treatment by anti–B-cell monoclonal antibodies.[32] The efficacy of anti–IL-6 antibody may be similar to that of anti-B-cell mAb, as complete remission was achieved in 61% of the 58 patients with BLPD treated with anti-B mAbs.[32] Anti–IL-6 mAb treatment was not effective in 4 patients (1 in SD, 3 in PD). However, for 3 of these 4 patients, improvements were achieved with another treatment (surgery in 1 patient and chemotherapy in 2 patients). Thus, the lack of efficacy of the anti–IL-6 mAb did not preclude the use of alternative therapies such as surgery and chemotherapy.

These encouraging data require confirmation in a larger group of patients, allowing statistical analysis and including patients with post-BMT BLPD, which is known to induce a poorer outcome.[32] It may also be of value to perform a phase 3 clinical trial in which anti–IL-6 antibody treatment could be compared to or associated with other forms of treatment (eg, anti-CD20 [anti-B cell]) mAbs or cytotoxic T lymphocytes in patients who have undergone BMT[12,29]).

Clonality, type of transplantation, and sites affected did not seem to influence treatment efficacy. The only factor that seemed to have a strong effect on efficacy was time to BLPD onset. These findings require confirmation in a larger series of patients. However, similar results were reported by Benkerrou et al,[32] who showed in a multivariate analysis that the late onset of BLPD was a major risk factor for the failure of anti-B-cell mAb treatment. Late-onset BLPD may be caused by a different physiopathogenic mechanism, with secondary oncogenic events (such as bcl-2 rearrangements, c-*myc*, n-ras, and p53 mutations) and LMP1 deletions[62-65] responsible for the formation of true lymphomas. In such cases, B-cell proliferation could be insensitive to anti–B-cell or anti–IL-6 antibody treatment but responsive to chemotherapy.

Our data, which are consistent with those obtained for SCID mice injected with B-cell lines derived from patients with BLPD,[48] suggest that IL-6 may, in some cases, have a major role in BLPD growth. Pathologic examination after treatment was possible in 4 patients in whom treatment was effective. In 2 patients, extensive tumor necrosis was observed. In one, infiltrating T cells and histiocytic cells replaced infiltrating B cells. In 2 other cases, complete resolution of BLPD was observed. No firm conclusions can be drawn from these data concerning the mechanism of BLPD resolution after anti–IL-6 antibody treatment. There are several possibilities. First, IL-6 may act as an autocrine–paracrine growth factor and may promote the growth of EBV-infected B cells. Indeed, IL-6 has been shown to promote the growth of EBV-infected B cells[45,46] and patients with BLPD produce abnormally high levels of IL-6.[45,47] A tumorigenic role for IL-6 in BLPD was also suggested by the results of experiments in which EBV-transformed B cells were transfected with human IL-6 cDNA: transfection significantly increased the proliferation of these cells in vivo and in vitro.[49,50] These observations suggest that original tumor cells (EBV-infected B cells), stromal cells, or both—as suggested for myeloma,[66] immunoblastic lymphoma,[40] and BLPD[47]—synthesize IL-6 that may act directly on the target EBV-infected B cells, promoting their growth. Alternatively, IL-6 has been shown to inhibit immune effector functions, such as natural killer (NK) cell activity, and cytotoxic functions of splenocytes in athymic mice, thereby permitting tumor development.[67] Therefore, NK cells, the function of which might be restored by anti–IL-6 antibody, may be involved in disease regression. However, it has been shown that beige–SCID mice, which show little NK activity, display a similar response to the anti–IL-6 antibody treatment of implanted EBV-B cell tumors (A.D. et al, unpublished data, 1997). These 2 mechanisms are not mutually exclusive and may both be involved in the potential efficacy of the anti–IL-6 monoclonal antibody.

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Improvement in Castleman's disease by humanized anti-interleukin-6 receptor antibody therapy

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Castleman's disease, an atypical lymphoproliferative disorder, can be classified into 2 types: hyaline-vascular and plasma cell types according to the histologic features of the affected lymph nodes. The plasma cell type is frequently associated with systemic manifestations and is often refractory to systemic therapy including corticosteroids and chemotherapy, particularly in multicentric form. Dysregulated overproduction of interleukin-6 (IL-6) from affected lymph nodes is thought to be responsible for the systemic manifestations of this disease. Therefore, interference with IL-6 signal transduction may constitute a new therapeutic strategy for this disease. We used humanized anti-IL-6 receptor antibody (rhPM-1) to treat 7 patients with multicentric plasma cell or mixed type Castleman's disease. All patients had systemic manifestations including secondary amyloidosis in 3. With the approval of our institution's ethics committee and the consent of the patients, they were treated with 50 to 100 mg rhPM-1 either once or twice weekly. Immediately after administration of rhPM-1, fever and fatigue disappeared, and anemia as well as serum levels of C-reactive protein (CRP), fibrinogen, and albumin started to improve. After 3 months of treatment, hypergammaglobulinemia and lymphadenopathy were remarkably alleviated, as were renal function abnormalities in patients with amyloidosis. Treatment was well tolerated with only transient leukopenia. Histopathologic examination revealed reduced follicular hyperplasia and vascularity after rhPM-1 treatment. The pathophysiologic significance of IL-6 in Castleman's disease was thus confirmed, and blockade of the IL-6 signal by rhPM-1 is thought to have potential as a new therapy based on the pathophysiologic mechanism of multicentric Castleman's disease. (Blood. 2000;95:56-61)

Introduction

Castleman's disease is a lymphoproliferative disorder with benign hyperplastic lymph nodes characterized histologically by follicular hyperplasia and capillary proliferation with endothelial hyperplasia.[1] Castleman's disease has been classified, according to the histopathologic findings, as either hyalinevascular or plasma cell type.[2,3] Patients with plasma cell type or a mixed hyaline-vascular and plasma cell type frequently have systemic manifestations such as fever, anemia, hypergammaglobulinemia, hypoalbuminemia, and an increase in acute phase proteins.[2-4] In cases of localized Castleman's disease, these clinical abnormalities may resolve after excision of the affected lymph nodes.[3-5] On the other hand, the multicentric form of Castleman's disease is often refractory to treatment even with corticosteroids or chemotherapy, and consequently the prognosis for such patients is poor. Infections are a common cause of death in multicentric Castleman's disease, as well as renal failure and other malignancies including malignant lymphoma and Kaposi's sarcoma.[6] Recently, Kaposi's sarcoma-associated herpesvirus (also called human herpesvirus type 8, KSHV/HHV-8) was reported to be an etiologic agent of Castleman's disease, especially in patients infected with human immunodeficiency virus (HIV).[7-9]

Interleukin-6 (IL-6) is a pleiotropic cytokine with a wide range of biologic activities, such as support of hematopoiesis, regulation of immune responses, and generation of acute phase reactions.[10] We previously demonstrated the generation of large quantities of IL-6 from the germinal centers of hyperplastic lymph nodes of patients with plasma cell type Castleman's disease.[5] We also showed a correlation between serum IL-6 concentration and clinical features, suggesting that dysregulated IL-6 production from affected lymph nodes may be responsible for the systemic manifestations of this disease. On the basis of these findings, Beck et al[11] showed that the in vivo administration of murine anti-IL-6 monoclonal antibody (mAb) to a patient with Castleman's disease seemed to be therapeutically effective, thus confirming the in vivo function of IL-6 in this disease. However, such a therapeutic effect was transient, because of either the emergence of neutralizing human antibodies against murine anti-IL-6 mAb or the inability to attain serum concentrations of anti-IL-6 mAb sufficient to neutralize increasing IL-6 levels.[12] The therapeutic value of murine anti-IL-6 mAb for human patients thus remains limited. We therefore attempted to block IL-6 signal transduction by using mAb against the IL-6 receptor (IL-6R). Furthermore, to be effective as a therapeutic agent administered to patients in repeated doses, murine anti-IL-6R mAb, PM-1, was engineered to be a human antibody by grafting the complimentarity-determining regions from the murine anti-IL-6R mAb into human IgG, thereby

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creating a functioning antigen-binding site in a reshaped human antibody, rhPM-1.[13] We used this humanized anti-IL-6R mAb to treat 7 patients with the multicentric form of plasma cell or mixed type Castleman's disease.

Patients and methods

Patients

Seven patients in Osaka University Hospital were recruited, each of whom had lymphadenopathy at multiple sites, mild splenomegaly, and constitutional symptoms such as fever and fatigue. Laboratory findings included anemia, polyclonal hypergammaglobulinemia, hypoalbuminemia, and increased acute phase proteins. All patients had plasma cell or mixed type Castleman's disease, based on histologic examination of the biopsy specimens. The clinical characteristics of the patients are shown in Table 1. All patients had active disease at the start of anti-IL-6R mAb treatment, and 4 of them had previously been treated with prednisolone or immunosuppressive therapy for more than 3 months. Three patients had amyloidosis secondary to Castleman's disease, and 5 had lymphocytic interstitial pneumonia, based on histologic examination of the lung biopsy specimens. None of the patients had autoimmune disorders, such as rheumatoid arthritis or Sjögren syndrome, or malignancies. Both HIV and KSHV/HHV-8 were undetectable by serologic assays and polymerase chain reaction (PCR). All patients gave their informed consent for the anti-IL-6R mAb therapy under the auspices of an approval approved by the ethics committee of Osaka University.

PCR/Southern hybridization and serologic analysis of KSHV/HHV-8

The PCR/Southern hybridization analysis of KSHV/HHV-8 was performed as follows. Genomic DNA was extracted from peripheral blood mononuclear cells or frozen specimens of lymph nodes or both. The DNA extracted from peripheral blood mononuclear cells of a Japanese patient with acquired immunodeficiency syndrome-Kaposi's sarcoma (AIDS-KS) with KSHV/HHV-8 infection was also used as a positive control for KSHV/HHV-8. Each 50-μL reaction mixture for PCR contained 0.4 to 0.6 μg total DNA, 2.5 U Ex Taq DNA polymerase (Takara, Kyoto, Japan), dNTP (0.25 mM each), 25 mM TAPS buffer (pH 9.3), 50 mM KCl, 2 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and the primers (10 pmol each) the sequences of which were as follows: outer forward primer: 5′-atggcactcgacaagagtatagtgg-3′; outer reverse primer: 5′-cgttagcgtggggaataccaacagg-3′ (from nucleotide 633 to nucleotide 1552 in KSU 18 551)[14]; inner forward primer: 5′-ctatccaagtgcacactcgctgtcc-3′; inner reverse primer: 5′-ggaaccaaggctgataggatacaaagg-3′ (from nucleotide 828 to nucleotide 1288 in KSU 18 551). The PCR procedure consisted of 1 cycle at 94°C for 2 minutes, then 40 cycles at 94°C for 1 minute, at 58°C for 1 minute, and at 72°C for 2 minutes, and 1 cycle at 72°C extension for 5 minutes with a DNA thermal cycler, Gene Amp PCR system 2400® (Perkin Elmer, Norwalk, CT). First, the DNA samples were amplified with the pair of outer primers and then with the pair of inner primers. The PCR products (10 μL) were then electrophoresed in a 2.0% agarose gel containing ethidium bromide and visualized with UV light. The PCR products were transferred to a hybond N+ (Amersham Inc., Arlington Heights, IL), hybridized with a DIG-labeled KSHV/HHV-8 specific probe: 5′-tgttggtgtaccacatctactccaaaatat-3′, and colorimetrically detected using a DIG DNA Labeling and Detection Kit (Boehringer Mannheim, Germany) in accordance with the manufacturer's instructions.

Immunofluorescence assay was performed as previously described.[15] Briefly, BCBL-1 cells (10^6/mL), the body cavity-based lymphoma cell line that is latently infected with KSHV/HHV-8 but not Epstein-Barr virus, were treated with 20 ng/mL phorbol ester (TPA: Sigma, St. Louis, MO) for 48 hours to induce the viral lytic antigen. Uninduced and TPA-induced BCBL-1 cells were then washed in phosphate-buffered saline (PBS), spotted on slides (2×10^4 cells/well), air dried in a laminar flow hood, and fixed in acetone at −20°C for 10 minutes. All sera from the patients with Castleman's disease and the Japanese patient with AIDS-KS were stored at −20°C and heat inactivated at 56°C for 30 minutes before use. Fixed cells were incubated at 37°C for 30 minutes with human sera serially diluted 1:2 beginning at 10-fold dilution. After washing rigorously in PBS, the slides were incubated with fluorescein isothiocyanate-conjugated goat anti-human IgG (Dako, Copenhagen, Denmark) at 37°C for 30 minutes. The slides were counterstained with a 1:20 000 dilution of Evan's blue (Sigma) at room temperature for 5 minutes, washed, mounted with 50% (vol/vol) glycerol in PBS, and examined under a fluorescence microscope.

Administration of humanized anti-IL-6R mAb, rhPM-1

Humanized anti-IL-6R mAb, rhPM-1, (IgG1 class) was produced in Chinese hamster ovary cells and purified on protein A.[13] rhPM-1 retains its specificity for natural and recombinant human IL-6R with the same affinity as possessed by the original murine anti-IL-6R mAb, PM-1, and inhibits IL-6 function both in vitro and in vivo.[13,16] rhPM-1 was dissolved in PBS and stored at −20°C until administration. The appropriate amount of rhPM-1 was then diluted to a volume of 50 mL in saline and administered intravenously over a period of 1 hour only after a skin test using 100 μL of the antibody was negative. Increasing doses (1, 10, 50, and 100 mg/patient) of rhPM-1 were administered intravenously twice weekly to establish the maximal tolerated dose in every patient. The dose of 100 mg/patient was chosen as the maximal dose because higher doses exceeded permitted levels of contaminating DNA after purification, in compliance with the guideline for the manufacture and testing of mAb products for human use suggested

Table 1. Clinical profiles of 7 patients with multicentric form of Castleman's disease who received humanized anti-IL-6R Ab therapy

Patient	Age/ Sex	Disease Duration* (years)	Histologic Diagnosis†	Complications†	Total Amount of Anti-IL-6R Ab (mg)	Number of Administrations	Duration of Anti-IL-6R Ab Therapy (mo)	Concominant Therapy†	Serum IL-6 Concentration Before Therapy (pg/mL)‡
1	33/M	3	Mixed type	LIP	4561	67	11	Pred 10 mg	40.1
2	43/F	1	Plasma cell type	LIP	4060	46	10	(−)	93.0
3	59/F	1	Plasma cell type	Secondary amyloidosis	1661	35	4	(−)	44.1
4	23/M	1	Mixed type	Secondary amyloidosis LIP	1860	25	4	(−)	20.6
5	51/M	3	Plasma cell type	Secondary amyloidosis	1200	26	4§	Pred 10 mg	6.5
6	54/F	1	Plasma cell type	LIP	660	15	2	L-PAM 2 mg Pred 40 mg	8.4
7	49/F	1	Plasma cell type	LIP	410	10	1	CYC 50 mg Pred 20 mg	24.5

*Disease duration is defined as the period since diagnosis.

†Pred indicates prednisolone; CYC, cyclophosphamide; L-PAM, melphalan; mixed type, mixed plasma cell and hyaline vascular type; LIP, lymphocytic interstitial pneumonia.

‡Normal range of serum IL-6 is < 1 pg/ml.

§The patient received anti-IL-6R antibody therapy for a total of 4 months after a 2-month interval.

by the Ministry of Health and Welfare, Japan, although no dose-limiting toxicity was observed. Fifty to 100 mg rhPM-1 was then administered either once or twice weekly for 5 to 42 weeks as maintenance therapy until completion of the study and cessation of rhPM-1 production. Total amounts of rhPM-1 administered, the number of administrations, and the duration of rhPM-1 therapy for each patient are summarized in Table 1.

Serum IL-6 and soluble IL-6R (sIL-6R) levels

Serum IL-6 levels were determined with a chemiluminescent enzyme immunoassay (CLEIA).[17] The principle of this assay is based on 2-site immunometric reverse sandwich reaction with the aid of a Lumipulse 1200 (Fujirebio Inc., Tokyo, Japan). Briefly, the mixture of the sample and mouse anti-IL-6 mAb conjugated with alkaline phosphatase was incubated at 37°C for 10 minutes, and then added to particles covalently linked to a murine anti-IL-6 mAb that recognizes a different epitope than that recognized by the original mAb. After a 10-minute incubation at 37°C, the particles were separated magnetically and washed in buffer. Subsequently, the substrate solution, 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy) phenyl-1, 2-dioxetane disodium salt was added at 37°C, and the chemiluminescent signal was photoncounted after 5 minutes. The detection limit for serum IL-6 was 0.1 pg/mL.

sIL-6R was measured by means of a dissociation-enhanced fluoroimmunoassay (DELFIA® Pharmacia, Uppsala, Sweden), as previously described.[18] Briefly, 200 μL of sample was added to microstrips coated with 200 μL anti-human IL-6R mAb (MT18) (1 μg/mL), and incubated at 4°C, followed by a reaction with 200 μL europium-labeled PM-1 mAb (100 ng/mL) at room temperature for 15 minutes. Fluorescence in each well was measured with a 1230 ARCUS fluorometer (Pharmacia).

Serum rhPM-1 and anti-rhPM-1 antibody levels

Serum rhPM-1 levels were assessed by enzyme-linked immunosorbent assay (ELISA). Briefly, 100 μL recombinant human sIL-6R (1 μg/mL) was added to the wells of an immunoplate precoated with MT18 and incubated at room temperature for 2 hours. After washing, 100 μL serum was added and incubated for additional 2 hours. After washing, bound rhPM-1 was measured using alkaline phosphatase-conjugated goat anti-human IgG. The calorimetric reaction was measured with a microplate reader.

Serum anti-rhPM-1 antibody levels were also determined by ELISA. Serum was added to the wells coated with 100 μL rhPM-1 (5 μg/mL) and incubated for 2 hours. After washing, biotin-conjugated rhPM-1 was added and developed with alkaline phosphatase-conjugated to streptavidin.

Results

Efficacy of humanized anti-IL-6R mAb, rhPM-1

Data representative of patients with Castleman's disease treated with rhPM-1 are shown in Figure 1. The patient was a 51-year-old man who suffered from amyloidosis secondary to Castleman's disease (patient 5 in Table 1). His disease was refractory to steroid therapy and chemotherapy consisting of melphalan, vincristine, and doxorubicin hydrochloride. Immediately after the administration of 20 mg rhPM-1, fever and malaise disappeared, whereas CRP and fibrinogen started to decrease within 24 hours, but this treatment was insufficient to obtain a satisfactory therapeutic effect. Treatment with repeated doses of 50 mg rhPM-1 twice a week completely normalized serum CRP levels within 4 weeks; 8 weeks of therapy was required to increase hemoglobin and albumin levels. After 2 months of treatment, hypergammaglobulinemia and lymphadenopathy also improved. Extension of the interval between administrations caused a slight increase in CRP and a decrease in hemoglobin levels, but constitutional symptoms did not reappear. Two weeks after termination of the therapy, fever, malaise, and anemia recurred, and treatment with increased doses of prednisolone had little effect on his symptoms. Readministration of rhPM-1, however, was as effective as the first treatment course, evidenced by improvements in CRP and hemoglobin levels. The data confirmed that the improvement was due to rhPM-1 therapy but not influenced by concomitant therapy. A similar therapeutic effect was obtained by treatment with 100 mg rhPM-1 once a week, without any significant adverse events. Thus, a total dose of 100 mg rhPM-1 per week was determined to be an effective therapy.

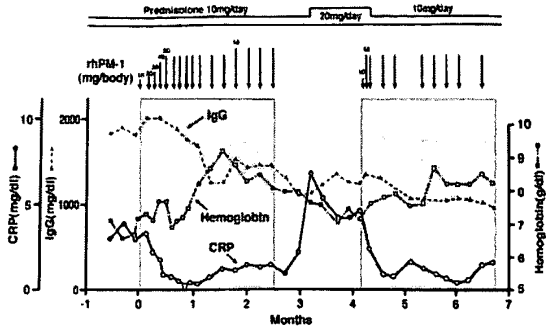

Figure 1. Representative changes in CRP, hemoglobin, and IgG concentrations in a patient with multicentric Castleman's disease during treatment with anit-IL-6R (rhPM-1). Arrows and shadings indicate the administration of rhPM-1. Note that treatment with rhPM-1 improved the clinical abnormalities, but that these recurred 2 weeks after the cessation of the rhPM-1 regimen. Readministration of rhPM-1 was as effective as the first treatment.

The patterns of response to rhPM-1 therapy are shown in Figure 2. Improvements in thrombocytosis, serum levels of CRP, fibrinogen, and serum amyloid A (SAA) protein were observed within 1 month in most cases, whereas hemoglobin, albumin, and immunoglobulin levels more gradually improved over a 3- to 4-month period. Two of the 7 patients showed increased levels of IgE (patient 2, 5300 IU/mL; patient 7, 650 IU/mL) before treatment, which were also reduced by rhPM-1 therapy (1100 IU/mL and 310 IU/mL, respectively). Autoantibodies (antinuclear antibody, anti-DNA antibody) observed in patients 2 and 6 disappeared after rhPM-1 therapy. Platelet counts and fibrinogen and immunoglobulin levels decreased only to the low-normal range. Similar therapeutic effects were observed in patients both with and without concomitant therapy. As a result of decrease in disease activity, it was possible to discontinue concomitant therapy in some patients. Importantly, these therapeutic effects did not diminish even after continuous 11-month treatment (patient 1). Therapy with rhPM-1 remarkably improved lymphadenopathy in all patients. Only in patient 4 were a few residual lymph nodes palpable, although their sizes were significantly reduced by the treatment. Computed tomography scanning confirmed the disappearance of pathologically significant visceral lymph nodes (> 1 cm in diameter) in all patients. During the treatment of patients 3, 4, and 5, who had secondary amyloidosis, constitutional symptoms such as appetite loss, constipation, and diarrhea (in all 3 patients) gradually improved. Bradyphasia and bradypragia, which might not be directly caused by the deposition of amyloid in the brain, also improved in patient 3. Furthermore, improvements in serum creatinine and daily urine protein levels were also observed after 4-month therapy in patient 3: before treatment, the serum creatinine level was 1.8 mg/dL, urine protein 3.1 g/d, serum β_2m 7.1 mg/L, and urine β_2 microglobulin 55.5 mg/d; after treatment, the corresponding values were 1.1 mg/dL, 0.9 g/d, 5.3 mg/L, and 7.4 mg/d. No major side effects of rhPM-1 therapy were observed, except for a transient and mild decrease in granulocyte counts on the day following mAb administration in 2 patients, which spontaneously

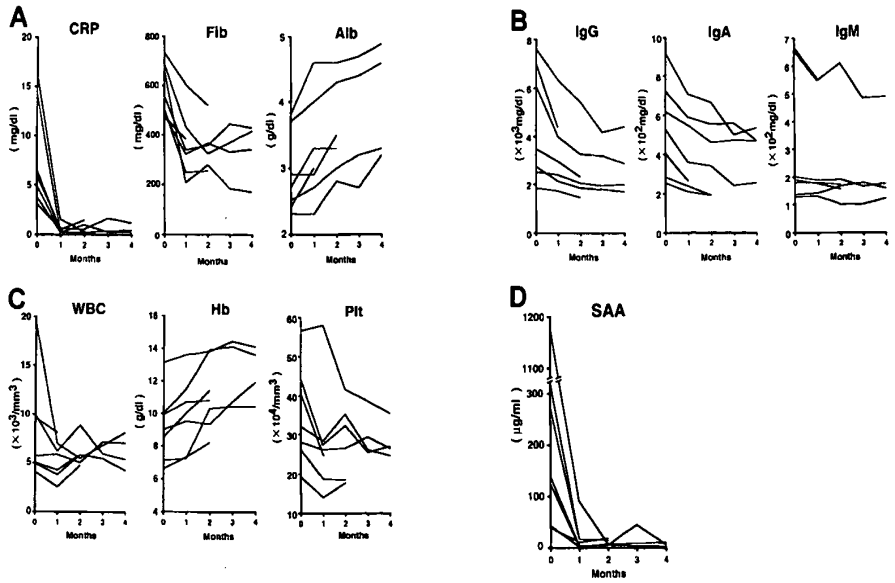

Figure 2. Clinical response patterns in 7 patients treated with rhPM-1. CRP indicates C-reactive protein; Fib, fibrinogen; Alb, albumin; IgG, immunoglobulin G; IgA, immunoglobulin A; IgM, immunoglobulin M; WBC, white blood cells; Hb, hemoglobin; Plt, platelets; and SAA, serum amyloid A.

recovered within 2 days. No decrease in T-cell function was observed, assessed either by a skin test with purified protein derivative of tuberculin or by a mixed lymphocyte culture test with allogeneic T cells.

Histologic examinations of affected lymph nodes

Therapy with rhPM-1 remarkably improved lymphadenopathy in all patients, and a few small residual lymph nodes remained palpable only in patient 4. To examine the histologic changes in the affected lymph nodes resulting from rhPM-1 therapy, a cervical lymph node that decreased in size on therapy was resected after 3 months of therapy in patient 4 and compared to pretreatment histopathologic appearance. Lymph nodes before rhPM-1 treatment showed features of a mixed hyaline-vascular and plasma cell type: (1) follicular hyperplasia with a large germinal center penetrated by branching hyaline blood vessels, (2) concentric appearance of mantle zone lymphocytes, and (3) proliferation of hyaline capillaries and numerous plasma cells in the interfollicular areas (Figure 3A and C). However, after treatment there was (1) a reduction in the size of lymph nodes, (2) a reduction in both the number and the size of follicles, resulting in a relative increase in the interfollicular areas (Figure 3B), and (3) a reduction in the vascularity of germinal centers with residual eosinophilic deposits (Figure 3D). However, the number of plasma cells and the extent of vascularity in the interfollicular areas did not show significant changes (Figure 3B).

Quantification of these changes was attempted by measuring both the longitudinal and transverse diameters of the germinal

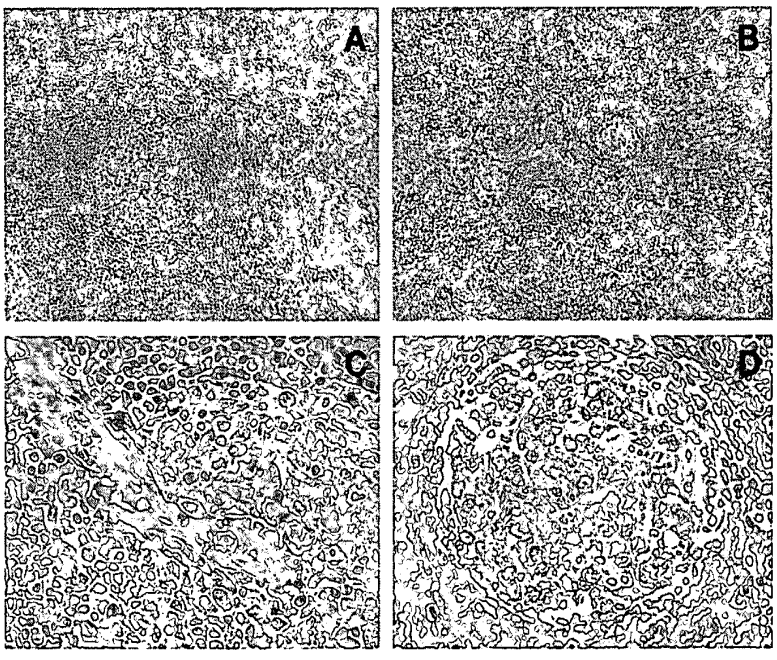

Figure 3. Serial histopathologic changes with rhPM-1 therapy. Hyperplastic lymph follicle with hyalinous capillary vessels penetrates into the germinal center before rhPM-1 treatment (A and C). Note reduction in both the size of lymph follicles and in the vascularity of germinal centers with residual eosinophilic deposits after rhPM-1 therapy (B and D). (Hematoxylin-eosin, A and B, original magnification ×100; C and D, original magnification ×400).

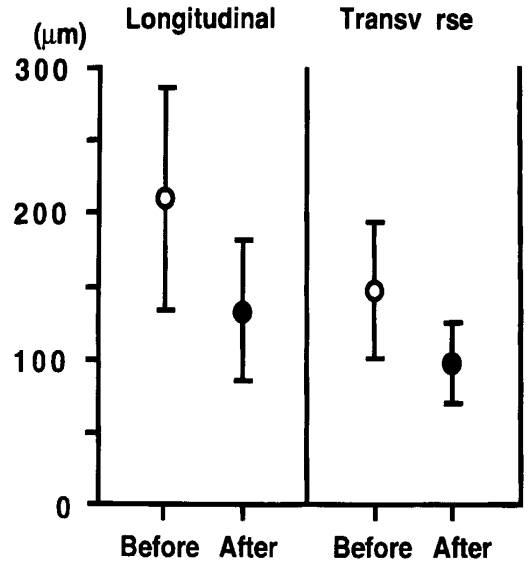

Figure 4. Changes in longitudinal and transverse diameters of the germinal centers. Open circles indicate mean diameters of germinal centers before treatment (n = 109) and closed circles indicate those after treatment (n = 15). The points and vertical bars indicate mean and SDs, respectively. Both the longitudinal and transverse diameters significantly decreased ($P < 0.005$, $P < 0.001$, respectively).

centers. As shown in Figure 4, both the longitudinal (210 ± 76 μm, mean ± SD, n = 109) and transverse diameters (150 ± 45 mm, n = 109) of the germinal centers before therapy were markedly reduced (133 ± 48 mm, and 98 ± 27 mm, n = 15, respectively) after therapy ($P < 0.005$ and $P < 0.001$, respectively, assessed by Mann-Whitney's U test).

Serum IL-6 and sIL-6R levels

Serum IL-6 levels did not change essentially, although they showed fluctuations unrelated temporally to the rhPM-1 treatment, whereas sIL-6R levels increased in the first month after the administration of rhPM-1, with peak serum levels of up to 700 ng/mL. Repeated administration of rhPM-1 uniformly and rapidly reduced the sIL-6R levels to normal in all patients. The maximum concentrations of serum sIL-6R did not increase with rhPM-1 maintenance therapy.

Serum rhPM-1 and anti-rhPM-1 antibody levels

The trough level of serum rhPM-1 was 10 μg/mL during maintenance therapy using 50 mg rhPM-1 twice a week and decreased to 5 μg/mL using treatment at 100 mg rhPM-1 once a week. Because a slight increase in CRP was observed with the latter regimen, the former schedule was considered to be more effective. There were no differences in responses in terms of constitutional symptoms observed between the 2 regimens. Ten μg/mL rhPM-1 had previously been proven to inhibit the IL-6-induced proliferation of myeloma/plasmacytoma cells in vitro.[16]

No serum anti-rhPM-1 antibodies were detected during the course of therapy of these 7 patients.

Discussi n

The clinical course of multicentric Castleman's disease is unpredictable, but the prognosis is generally poor.[4,6] The accompanying constitutive inflammatory state may result in complications such as pneumonia, amyloidosis, and malignancies, and treatment should therefore be introduced as early as possible. Although spontaneous remissions are observed in some patients,[4,6] in other patients the disease is refractory to conventional therapies such as corticosteroids, cytotoxic agents, or radiation. Thus, a new therapeutic strategy for multicentric Castleman's disease is needed.

The present study showed that the humanized anti-IL-6R mAb, rhPM-1, can achieve marked responses in the refractory form of this disease without significant adverse reactions or development of neutralizing antibodies. Furthermore, rhPM-1 therapy also improved the symptoms and biochemical abnormalities of secondary amyloidosis, which is often otherwise untreatable at present, although successful therapy to reduce the supply of amyloid fibril protein precursors is followed by substantial regression of amyloid.[19] Although re-biopsy of the kidney was not performed after rhPM-1 therapy, reduced serum creatinine and urine protein levels suggest that rhPM-1 therapy may alleviate renal amyloidosis. In addition, rhPM-1 therapy improved the hydronephrosis in patient 3 who, before therapy, required nephrostomy due to ureterostenosis resulting from amyloidosis of the ureter. These findings suggest that visceral amyloid deposition in organs may also be reversible, if SAA production is inhibited by blocking IL-6 signaling. rhPM-1 may also reduce the likelihood of malignant lymphoma and Kaposi's sarcoma because IL-6 is a potent growth factor for these malignancies.[20,21]

The fact that the blockade of the IL-6 signal by humanized anti-IL-6R mAb improved the systemic manifestations of Castleman's disease confirms a central role for IL-6 in the pathogenesis of this disease. In particular, overproduction of IL-6 causes inflammatory symptoms, such as low-grade fever and generalized malaise, as well as laboratory findings, such as leukocytosis, anemia, thrombocytosis, hypergammaglobulinemia, hypoalbuminemia, and an increase in CRP, fibrinogen, and SAA levels. The observation that elevation in serum IgE levels also resolved provides further evidence that IL-6 plays some role in regulating IgE production in vivo and supports the view that IL-6 induces differentiation of naive T cells into Th2 cells in vitro.[22] Autoantibodies frequently observed in this disease also disappeared during rhPM-1 treatment, suggesting that IL-6 may also mediate autoimmune phenomena.

Histologic changes observed in the lymph nodes after treatment imply an important pathogenic mechanism of this disease. That is, IL-6 may be involved in the follicular hyperplasia of affected lymph nodes because blockade of the IL-6 signal reduced both their number and size. Because IL-6 is mainly produced by activated B cells in the germinal center of lymph follicles,[5,11] an autocrine growth mechanism may be mediated by IL-6 in follicular B cells of patients with this disease. The disappearance of hyaline capillaries in the follicles after treatment with rhPM-1 indicates that IL-6 may also play a role in angiogenesis, although it remains to be determined whether or not IL-6 acts directly on endothelial cells. IL-6 may promote angiogenesis through the induction of other factors such as vascular endothelial growth factor,[23] because patients with a typical hyaline-vascular variant of Castleman's disease do not always show an increase in serum IL-6 levels.

We have shown the efficacy of humanized antibodies for anti-cytokine therapy because no anti-rhPM-1 antibodies were detectable, even in the patient who received multiple injections (67 doses) for 11 months. Klein et al[12] reported that anti-IL-6 mAb therapy induced a continuous increase in IL-6 production in vivo, and consequently was unable to administer sufficient anti-IL-6 mAb to neutralize IL-6 activity. A similar phenomenon was observed during anti-IL-6R mAb therapy as serum sIL-6R increased after administration of rhPM-1. However, this tendency did

not continue, and the highest concentration observed was about 700 ng/mL, with trough levels of the serum rhPM-1 concentration of 5 to 10 μg/mL. Moreover, maximum sIL-6R levels gradually decreased with repeated administrations of rhPM-1 over 2 months. This may be the reason the efficacy of humanized anti-IL-6R mAb therapy did not diminish over time. On the other hand, serum IL-6 levels did not change essentially although they showed some fluctuations. This is probably because rhPM-1 reacts with IL-6R and interferes with IL-6-binding to IL-6R, but does not react with IL-6 itself. It may also be the reason a relatively prompt relapse occurred (after 2 weeks) when the treatment was stopped. Two weeks is consistent with the period when rhPM-1 was detectable in blood after termination of the therapy. However, serum IL-6 levels may decrease if we continue the treatment further, because lymphadenopathy improved during the treatment and a reduction in both the number and size of the follicles, which are the main sources of IL-6 in this disease,[5,11] was observed in a lymph node.

Although rhPM-1 therapy improved the symptoms associated with Castleman's disease and reduced lymphadenopathy, recurrence of the disease was observed after termination of this therapy. Our data indicate that overproduction of IL-6 causes most of the abnormalities of Castleman's disease, but the cause of the constitutive overproduction of IL-6 is at present unknown. This may be due to a lack of NF-IL6, resulting from genetic abnormalities,[24] although familial aggregation has not been reported. Because Castleman's disease is frequently associated with HIV infection[6] and HIV infection also causes overproduction of IL-6,[25] infection with a retrovirus may also play a role in pathogenesis. In this regard, KSHV/HHV-8 was recently reported to be frequently positive in multicentric Castleman's disease, especially in HIV-infected patients.[7-9] Because a homologue to human IL-6, viral IL-6 (vIL-6), has been identified in the KSHV/HHV-8 genome,[26,27] which retains biologic activities similar to human IL-6, KSHV/HHV-8 is suspected of being involved in the pathogenesis of Castleman's disease. Although serologic and PCR assays in our patients were negative for HIV and KSHV/HHV-8, infection with another organism that can promote IL-6 production might be involved in this disease.

Further studies will be required to identify the etiology of the deregulated production of human IL-6 in Castleman's disease. It is also necessary to examine the effect of rhPM-1 therapy on IL-6 gene, steroid-responsive gene, and multidrug-resistant gene expression in germinal center cells because incorporation of immunotherapy may cause the emergence of drug resistance as a consequence of perturbations in intracellular signaling and the population kinetics of tumor cell adaptation and selection. Nevertheless, the pathophysiologic significance of human IL-6 is confirmed by this study because the clinical manifestations of Castleman's disease are alleviated by blockade of human IL-6R. The present study, therefore, demonstrates a novel and promising therapy, based on blockade of IL-6 signaling with a humanized anti-IL-6R antibody, which may also be effective for other diseases characterized by overproduction of IL-6.

Acknowledgments

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Anti-interleukin 6 receptor antibody treatment in rheumatic disease

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Abstract

Interleukin 6 (IL6) is a pleiotropic cytokine with a wide range of biological activities. IL6 transgene into mice gives rise to the abnormalities such as hyper-γ-globulinaemia, thrombocytosis, infiltration of inflammatory cells into the tissues, mesangial cell proliferation of the kidney as well as splenomegaly and lymphadenopathy, which are predictable by the biological functions of IL6 shown in vitro. Continuous overproduction of IL6 is observed in patients with some immune-inflammatory diseases such as Castleman's disease and rheumatoid arthritis that are frequently associated with similar abnormalities to those of IL6 transgenic mice, strongly suggesting the involvement of IL6 in the human diseases. Successful treatment of the model animals for immune-inflammatory diseases with anti-IL6 receptor (IL6R) antibody thus indicates the possible application of IL6 blocking agents to treat the IL6 related immune-inflammatory diseases of humans. In this review, the new therapeutic strategy for Castleman's disease and RA using humanised antibody to human IL6 receptor, MRA, is discussed.

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As each monoclonal antibody grasps a specific target antigen epitope, it has been widely used as a research device or a diagnostic drug. It was also expected as a "magic bullet" to treat cancer patients, but the attempt to apply monoclonal antibodies to treat cancer patients fell out of favour because of the failure caused by unexpected toxicity in some clinical trials in the late 1980s.[1] Similarly, clinical trials of several antibodies targeted against the blood infection sepsis were halted because the treated patients were found to be more likely to die than untreated patients in the early 1990s.[1] However, after accumulation of knowledge about the function and distribution of target molecules and also after working out new strategies to use chimerised or humanised antibodies of which varying amounts were replaced with human antibody sequences, therapeutic antibodies have begun to be applied to treat patients not only with cancers but also with immune-inflammatory disorders or acute rejection after organ transplantation. Interleukin 6 (IL6) is a pleiotropic cytokine that regulates immunological reactions in host defence, inflammation, haematopoiesis, and oncogenesis.[2 3] Although IL6 plays important parts in the host immune system, dysregulated overproduction of IL6 causes unfavourable clinical symptoms and laboratory findings in the patients with immune-inflammatory diseases. In this review, pathological significance of IL6 overproduction in immune-inflammatory diseases and the therapeutic approach by blocking IL6 function using humanised anti-IL6 receptor (IL6R) antibody, MRA, are discussed.

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Rationale for IL6 blocking treatment in immune-inflammatory diseases

PREIOTROPIC FUNCTIONS OF IL6

IL6 was originally identified as a B cell differentiation factor (BCDF) in the culture supernatants of mitogen or antigen stimulated peripheral mononuclear cells, which induced B cells to produce immunoglobulin[4 5] and the cDNA encoding human IL6 was cloned in 1986.[6] IL6 was then proved to be a pleiotropic cytokine with a wide range of biological activities (fig 1) and produced by various types of lymphoid and non-lymphoid cells, such as T cells, B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, mesangial cells, and several tumour cells.[5] In addition to BCDF activity, IL6 induces T cell growth and cytotoxic T cell differentiation[7–12] through augmentation of IL2 receptor expression[5] and IL2 production.[13] IL6 acts synergistically with IL3 on haematopoiesis to support the formation of multi-lineage blast cell colonies,[14–16] suggesting that it prompts haematopoietic stem cells in the G_0 phase to enter into the G_1 phase. Furthermore, IL6 is a potent inducer of terminal macrophage differentiation[17] and osteoclast differentiation,[20] and also induces platelet production by functioning as a megakaryocytic differentiation factor.[21–23] In the acute phase reaction, IL6 stimulates hepatocytes to produce acute phase proteins such as C reactive protein (CRP), fibrinogen, α_1 antitrypsin, and serum amyloid A (SAA)[24–25] while it simultaneously suppresses the albumin production.[26] Leucocytosis and fever have been found to be the result of in vivo administration of IL6.[27] As a cell growth factor, IL6 can induce the proliferation of mesangial cells,[28] epidermal keratinocytes,[29 30] and various tumour cells such as plasmacytoma cells,[31] multiple myeloma cells,[32] and renal carcinoma cells.[33]

ABNORMAL FINDINGS OBSERVED IN IL6 TRANSGENIC MICE

From a study using IL6 transgenic mice, we can learn what kinds of abnormalities IL6 hyperproduction causes in vivo. Transgenic mice were produced by introduction of the human IL6 gene under the transcription control of the immunoglobulin μ heavy chain

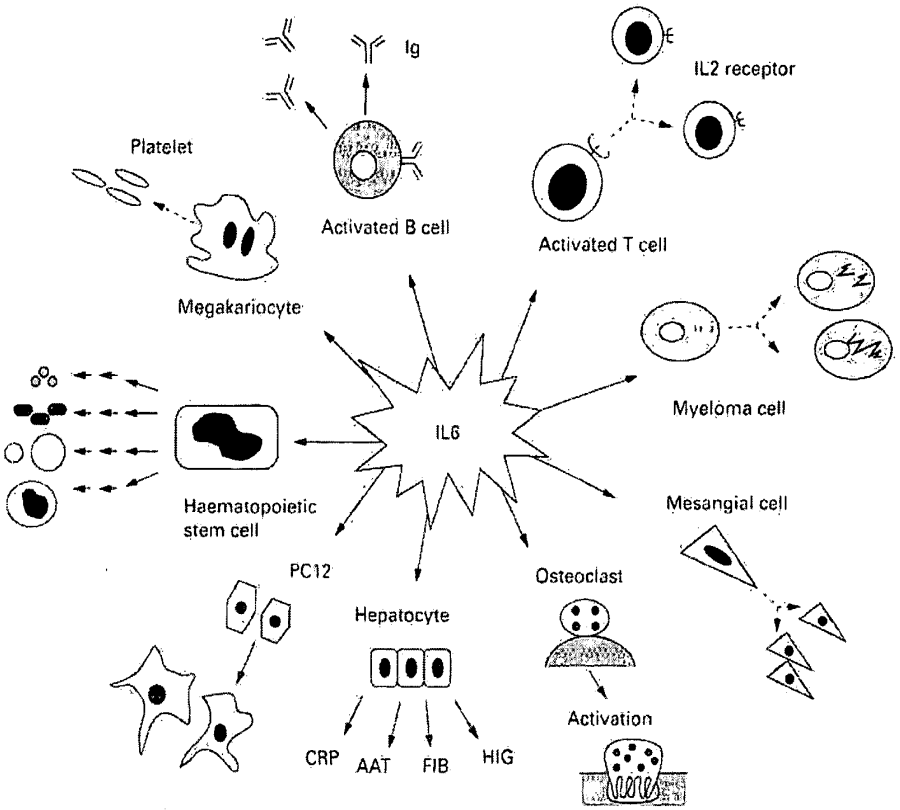

Figure 1 Schematic model of IL6 functions. IL6 is a pleiotropic cytokine with a wide range of biological activities.

enhancer (Eμ) into C57BL/6 mice.[31] Polyclonal hyper-γ-globulinaemia with massive plasmacytosis in the spleen and lymph nodes was then observed in Eμ-IL6 transgenic mice (fig 2, table 1). They showed increased levels of serum fibrinogen and reduced levels of serum albumin as well as an increased number of megakaryocytes in the bone marrow. IL6 transgenic mice also showed mesangial cell proliferation in the glomerulus (fig 3A), and lymphocyte proliferation with plasma cell infiltration in the interlobular septa and adjacent alveolar septa in the lung (fig 3C), and died within four or five months. These data indicate that IL6 can function in vivo in the same way as it does in vitro. Furthermore, these abnormal findings may represent some of the features observed in human immune-inflammatory disease such as Castleman's disease and rheumatoid arthritis (RA), and the patients with active disease are always associated with overproduction of IL6 in vivo.[35-39]

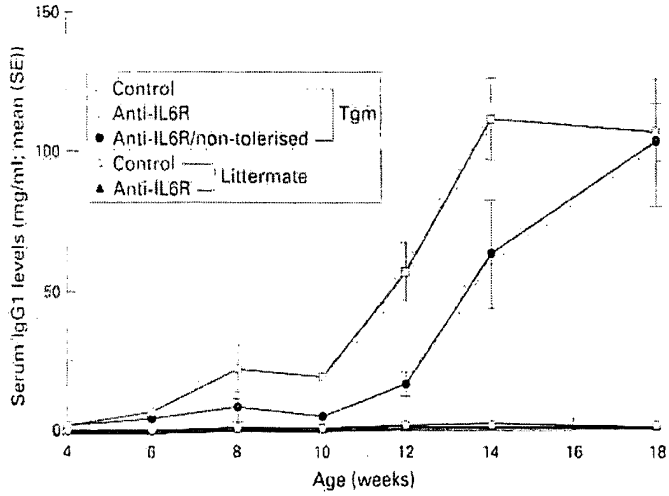

Figure 2 Anti-IL6R antibody treatment suppressed the increase in serum IgG1 levels in IL6 transgenic mice. C57BL/6J human IL6 transgenic mice (tgm) were first intravenously treated with 2 mg/body of rat antimouse IL6R antibody (MR16-1) at 4 weeks of age to induce tolerance to the mice against rat IgG, then subcutaneously treated with 100 ug/body twice weekly from 5 to 17 weeks of age. Serum IgG1 levels were analysed by ELISA.

ANTI-IL6R ANTIBODY TREATMENT IN ANIMAL MODELS

The IL6R complex comprises two functional different membrane proteins: an 80 kDa ligand binding chain (IL6R, IL6R α-chain, CD126) and a 130 kDa non-ligand binding but signal transducing chain (gp130, IL6R β-chain, CD130). IL6R has a very short intracytoplasmic portion containing only 82 amino acids that is not essential for signal transduction. IL6, when bound to either a membrane anchored or soluble form of IL6R (sIL6R), induces the homodimerisation of gp130, resulting in a high affinity functional receptor.[30] Therefore, several approaches can be proposed to block IL6 signal transduction: (1) inhibition of IL6 production or neutralisation of IL6; (2) blockade of IL6 binding to IL6R; (3) blockade of IL6/IL6R complex binding to gp130; (4)

Table 1 Abnormal findings in IL6 transgenic mice (C57 Bl/6J)

Plasmacytosis (no evidence of monoclonality)
Splenomegaly
Lymphadenopathy
Megakaryocytosis in the bone marrow
Mesangial proliferative glomerulonephritis (MPGN)

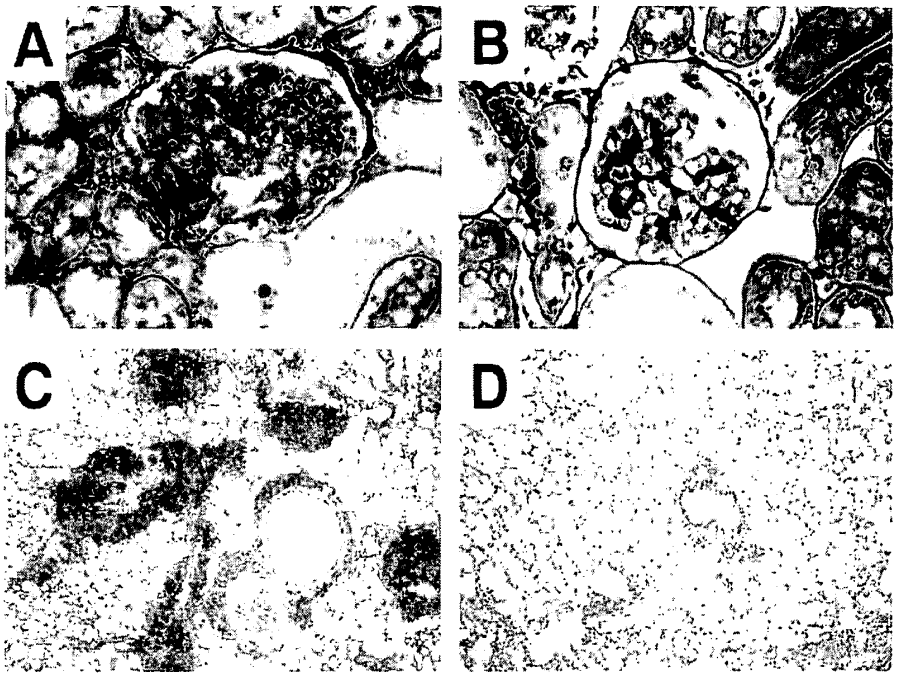

Figure 3 Anti-IL6R antibody treatment inhibited the development of mesangial proliferative glomerulonephritis and lymphocytic interstitial pneumonia in IL6 transgenic mice. C57BL/6J human IL6 transgenic mice (tgm) were treated with rat antimouse IL6R antibody (MR16–1) similarly to that of figure 2. Left kidneys and lungs were removed and fixed with 20% neutral buffered formalin for histological examination.

blockade of the intracytoplasmic signal through gp130. We have used anti-IL6R antibody to block IL6 signal transduction as a therapeutic strategy. This concept was tested in animal models: IL6 transgenic mice and collagen induced arthritis (CIA) mice.

As we described above, Eμ-IL6 transgenic mice developed hyper-γ-globulinaemia with massive plasma cell infiltration in the spleen and lymph nodes. Treatment with rat antimouse IL6R antibody, MR16–1, completely inhibited the increase in serum γ-globulin levels (fig 2) as well as plasmacytosis. The mice also suffered from mesangial proliferative glomerulonephritis and they started to show proteinuria and haematuria at the age of 8–10 weeks. MR16–1 treatment prevented the occurrence of proteinuria and haematuria in the mice. It was confirmed by the histological findings that mice treated by MR16–1 showed normal glomerulus (fig 3B) while untreated mice showed mesangial cell proliferation with occlusion of the glomerular capillaries (fig 3A). The IL6 transgenic mice also showed lymphocyte infiltration in the many organs and developed the histological feature characteristic to lymphocytic interstitial pneumonia (LIP) in the lung (fig 3C). However, MR16–1 treatment completely prevented the pathological change of lung (fig 3D). Similarly, all the other abnormalities such as increase in serum fibrinogen, SAA and platelet counts as well as decrease in serum albumin observed in the IL6 transgenic mice were ameliorated by the administration of MR16–1.

CIA in DBA/1J mice is an experimental arthritis model widely used for the evaluation of therapeutic agents for human RA. As inactivation of the IL6 gene completely protected DBA/1J mice from CIA[41] or it delayed the onset of CIA and also reduced the severity,[42] IL6 seems to be required for the development of CIA. Antimouse IL6R antibody treatment also suppressed the development of arthritis and protected knee joints from destructive change in DBA/1J mice immunised with bovine type II collagen.[43] Amounts of 0.5 mg, 2 mg, or 8 mg of rat antimouse IL6R antibody, MR16–1, were administered into DBA/1J mice once on the day of the first collagen immunisation. This MR16–1 treatment prevented the development of CIA in a dose dependent manner assessed by a visual scoring system for arthritis in four limbs. Although MR16–1 treatment on the day 3 after the immunisation similarly suppressed the development of arthritis, anti-arthritis effects were not observed when the same dose of MR16–1 was administered on the day 7 or later.[43] Taken together with the findings that treatment of CIA with anti-TNFα and anti-IL1 antibodies has been shown to be effective on established murine CIA,[44 45] IL6 may act earlier in the course of CIA than that of TNFα and IL1.

As a monoclonal antibody recognises a specific antigen epitope, a monoclonal antibody to human antigen epitope does not always cross react with that of other species. The safety and in vivo effect of humanised antihuman IL6R antibody, MRA, on the development of CIA were thus examined in cynomolgus monkeys (Macaca fascicularis) because MRA cross reacts with their IL6R[46] but not with that of murine. Joint swelling and stiffness as well as limb oedema appeared at four weeks after the first immunisation with bovine type II collagen with complete adjuvant and increased both in the number and in the degree up to six to eight weeks. Although the inflammation of joints spontaneously decreased, destructive changes

remained in the joints. Intravenous administration of MRA (10 mg/kg, once a week) significantly inhibited the onset of joint inflammation as well as the increase of serum CRP, fibrinogen levels and erythrocyte sedimentation rate (ESR) in the monkeys immunised with type II collagen. Histological examination at 14 weeks revealed that pathological changes did not occur in the MRA treated group while synovial proliferation, pannus formation, infiltration of neutrophils, angiogenesis, and cartilage and bone destruction were observed in the joints of the control group monkeys, most of which looked closely like the pathological changes of human RA joints. These findings clearly demonstrate that MRA prevented the development of CIA in cynomolgus monkey. Furthermore, administration of MRA did not affect blood cell, liver, kidney or heart in the cynomolgus monkey.

These data suggest that MRA can be a therapeutic agent for the treatment of human RA.

PATHOLOGICAL SIGNIFICANCE OF IL6 IN CASTLEMAN'S DISEASE

Castleman's disease,[47] an atypical lymphoproliferative disorder, can be classified into two types: hyaline-vascular and plasma-cell type according to the histological features of the affected lymph nodes.[48 49] The latter type is frequently associated with systemic manifestations characterised by chronic inflammatory symptoms such as fever, general fatigue, anaemia, an increased ESR, increased levels of CRP and fibrinogen[35 48-50] and immunological abnormalities such as polyclonal hyper-γ-globulinaemia, and the presence of autoantibodies. Patients with Castleman's disease frequently show lymphocytic interstitial pneumonia[51] and mesangial proliferative glomerulonephritis (MPGN), and sometimes develop autoimmune haemolytic anaemia as complications. Some of these findings resemble those seen in the IL6 transgenic mice. In cases of localised Castleman's disease, these clinical abnormalities may resolve after excision of the affected lymph nodes.[35 48-50] On the other hand, the multicentric form of Castleman's disease is often refractory to treatment even with corticosteroids or chemotherapy, and consequently the prognosis of such patients is poor. Therefore, a new therapeutic strategy has been required. Recently, Kaposi's sarcoma associated herpesvirus (also called human herpesvirus type 8, KSHV/HHV-8) was reported to be an actiological agent of this disease, especially in human immunodeficiency virus (HIV) infected patients.[52-54]

We previously demonstrated constitutive overproduction of IL6 from the germinal centres of hyperplastic lymph nodes and a correlation between serum IL6 levels and clinical abnormalities of patients with plasma-cell type Castleman's disease. And then we suggested that dysregulated IL6 production from affected lymph nodes might be responsible for the systemic manifestations of this disease.[35]

Beck *et al*[55] showed that the in vivo administration of murine anti-IL6 neutralising antibody to a patient with Castleman's disease seemed to be therapeutically effective, thus confirming the in vivo function of IL6 in this disease. However, such a therapeutic effect might be transient, either because of the emergence of neutralising human antibodies against murine anti-IL6 antibody or because of the inability to attain serum concentrations of anti-IL6 monoclonal antibody sufficient to neutralise increasing IL6 levels.[56] Therefore, the therapeutic value of murine anti-IL6 antibody for human patients remained limited and then humanised anti-IL6R antibody treatment can constitute a new therapeutic strategy for patients with multicentric Castleman's disease.

PATHOLOGICAL SIGNIFICANCE OF IL6 IN RA

RA is a typical immune-inflammatory disease characterised by persistent synovitis with synovial cell proliferation and destructive changes in bone and cartilage of multiple joints. Emergence of rheumatoid factors and increase in platelet counts, γ-globulin, CRP, ESR, and serum amyloid A protein levels and decrease in serum albumin may be explained by uncontrolled IL6 overproduction.[57] As IL6 in the presence of soluble IL6R activates osteoclasts to induce bone absorption in vitro,[20] and also as anti-IL6 antibody treatment prevented the osteoporosis in vivo in ovariectomised rats,[58] IL6 may be involved in the osteoporosis and bone and cartilage destruction of RA. As IL6 can upregulate the expression of intercellular adhesion molecule (ICAM)-1,[59] it may recruit immunocompetent cells into the inflammatory tissues. Large amounts of IL6 were indeed observed both in the sera and synovial fluids from the affected joints of RA patients.[30-39] Taken together, these findings indicate that it is probable that IL6 plays important parts in the pathogenesis of RA. Wendling *et al* reported that the administration of mouse monoclonal anti-IL6 antibody to the patients with RA resulted in improvement of symptoms and laboratory findings of RA although the effects were transient.[60] The fact confirmed that IL6 plays an important part in this disease.

Treatment of Castleman's diseases and RA with humanised anti-IL6R antibody, MRA

HUMANISED ANTI-IL6R ANTIBODY

To be effective as therapeutic agents administered to patients in repeated doses, mouse antibodies must therefore be engineered to look like human antibodies. Reshaped human PM-1 (rhPM-1) is constructed by grafting the complementarity determining regions (CDR) from mouse PM-1, a specific monoclonal antibody against human IL6R, into human IgG to recreate a properly functioning antigen binding site in a reshaped human antibody.[61] rhPM-1 is equivalent to both mouse and chimeric PM-1 in terms of antigen binding and growth inhibition of myeloma cells in vitro.[61 62] It looks very much like a human antibody and can therefore be expected to have less immunogenicity in vivo in human. rhPM-1 is now named MRA.

We have attempted to inhibit IL6 signal transduction by using this humanised antibody against the human IL6R.

TREATMENT OF CASTLEMAN'S DISEASE

We used humanised anti-IL6 receptor antibody, MRA, to treat seven patients with multicentric plasma-cell type or mixed type Castleman's disease.[63] All patients had active disease at the start of MRA treatment, and four of them had previously been treated with prednisolone or immune suppressive agents for more than three months. Three patients had amyloidosis secondary to Castleman's disease, and five had lymphocytic interstitial pneumonia. After obtaining their informed consent and an approval of the Ethical Committee of Osaka University, 50–100 mg of MRA was administered either once or twice weekly. The trough level of serum MRA was 10 μg/ml during maintenance treatment using 50 mg MRA twice a week, and decreased to 5 μg/ml using treatment at 100 mg MRA once a week. There were no differences in responses in terms of constitutional symptoms observed between the two regimens. Ten μg/ml of MRA had previously been proved to inhibit the IL6 induced proliferation of myeloma/plasmacytoma cells in vitro.[62] Treatment was well tolerated except for a transient and mild decrease in granulocyte counts on the day after MRA administration in two patients that spontaneously recovered within two days. No decrease in T cell function was observed, assessed either by a skin test with purified protein derivative of tuberculin or by a mixed lymphocyte culture (MLC) test with allogeneic T cells. Representative data of the MRA treatment for Castleman's disease are shown in figure 4. Immediately after the MRA administration, fever and fatigue disappeared, and anaemia as well as serum levels of CRP, fibrinogen, SAA and albumin started to improve. After three months of treatment, hyper-γ-globulinaemia and lymphadenopathy were also remarkably alleviated, as were renal function abnormalities in patients with amyloidosis. Autoantibodies such as antinuclear antibody and anti-DNA antibody disappeared after MRA treatment. Histopathological examination of lymph nodes revealed reduction in follicular hyperplasia and vascularity after MRA treatment. The findings imply an important pathogenic mechanism of IL6 in the follicular hyperplasia of affected lymph nodes as well as infiltration of hyaline capillaries with endothelial cell proliferation. These data showed humanised anti-IL6R monoclonal antibody, MRA, can achieve marked responses in the refractory form of this disease without significant adverse reactions or development of neutralising antibodies. It also confirmed the pathophysiological significance of IL6 in Castleman's disease, and blockade of the IL6 signal by MRA may have potential as a new treatment based on the pathophysiological mechanism of multicentric form Castleman's disease.

TREATMENT OF RA

Before the clinical trial of humanised anti-IL6R antibody, MRA, we compassionately treated 11 patients with refractory RA by MRA after obtaining the permission of ethical committee of our institute.[57] Before MRA treatment, they all had active disease resistant to any conventional treatment using various disease modifying antirheumatic drugs (DMARDs) including methotrexate, and corticosteroids. The patients were treated with MRA in the same regimen as that for Castleman's disease. The treatment was well tolerated and no major side effect was observed except an appearance of anti-idiotypic anti-

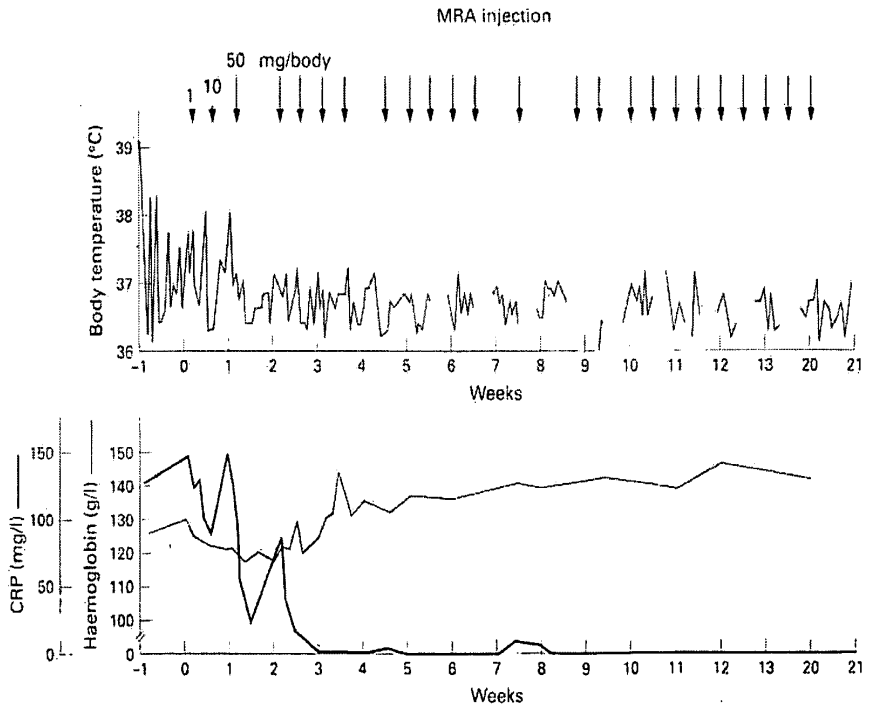

Figure 4 Improvement in Castleman's disease by humanised anti-IL6R antibody treatment.

body in one case, who was therefore withdrawn. Two additional patients were withdrawn: one was because of occurrence of angina attack the relation of which to MRA administration could not be classified, and the other was because of her private reasons. A transit decrease in neutrophil counts mostly within normal range was observed in most of the cases on the next day after MRA administration similarly to Castleman's disease patients. In the eight patients who received the MRA treatment for more than eight weeks, both clinical and biological improvement appeared during the treatment. Representative data of a patient treated with MRA are shown in figure 5. At eight weeks, clinical response was 88% assessed by ACR20 criteria, and 50% by ACR50. Similar response was obtained in the four patients who received MRA treatment for 24 weeks (unpublished data). These results indicate that MRA is relatively safe and useful for the treatment of RA. On the basis of our data, phase I/II clinical trial of MRA in Japan and phase I trial in the United Kingdom are now in progress.

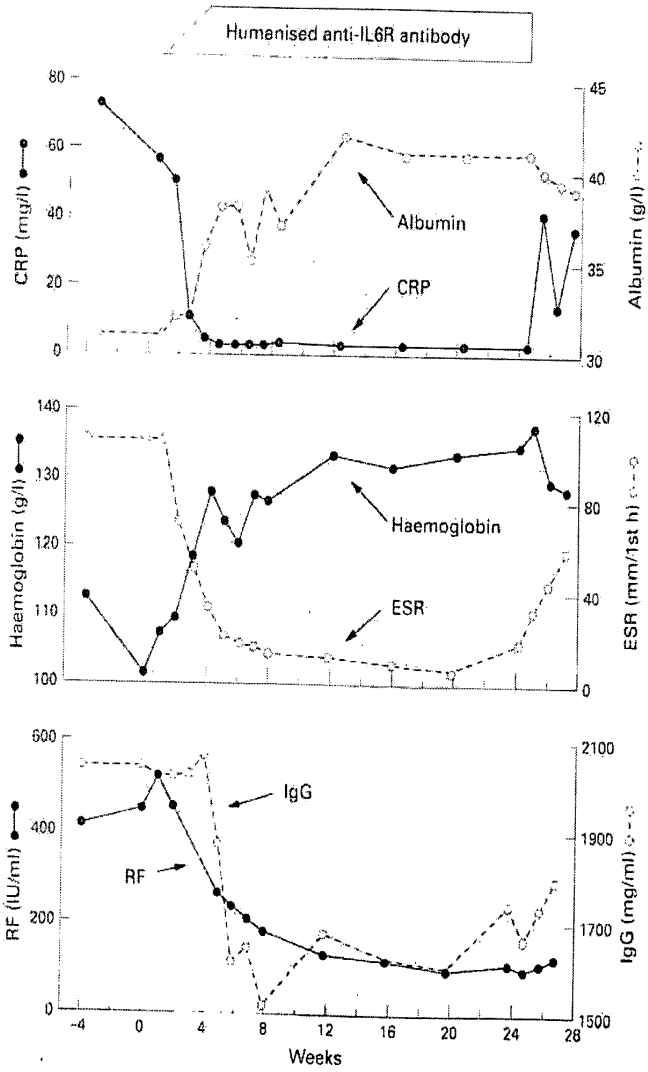

Figure 5 Improvement in RA by humanised anti-IL6R antibody treatment.

Conclusion

Recent progress in the study of immune-inflammatory diseases revealed the pathological significance of some proinflammatory cytokines in the diseases. In addition, the advances in molecular technology made it possible to constitute a new strategy to use humanised monoclonal antibody to modulate the cytokine signals. There are anti-cytokine treatments such as recombinant soluble TNF receptor-Fc fusion protein (Etanercept)[64] for RA and chimeric anti-TNFα monoclonal antibody (Infliximab) for RA[65,66] and Crohn's disease[67] already approved in the USA. IL1 receptor antagonist, which blocks IL1 function, and other cytokine inhibitors or even anti-inflammatory cytokine such as IL10 are now under evaluation for their safety and efficacy. Interference of IL6 signal transduction with a humanised anti-IL6 receptor antibody is one of these therapeutic approaches. Each of these agents is to be evaluated specifically for their long term safety and efficacy for these chronic diseases. As many proinflammatory cytokines and immunoregulatory cytokines seem to play an integral part in the pathogenesis of immune-inflammatory disease, combination treatment consisting of some agents may be more effective than that of a single agent. Studies are necessary to understand the intracellular events, such as signal transduction and gene regulation, in the cytokine network which physiologically regulates the overfunction of each cytokine in vivo.

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SOCS1 Is a Critical Inhibitor of Interferon γ Signaling and Prevents the Potentially Fatal Neonatal Actions of this Cytokine

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Summary

Mice lacking suppressor of cytokine signaling-1 (SOCS1) develop a complex fatal neonatal disease. In this study, *SOCS1*$^{-/-}$ mice were shown to exhibit excessive responses typical of those induced by interferon γ (IFNγ), were hyperresponsive to viral infection, and yielded macrophages with an enhanced IFNγ-dependent capacity to kill *L. major* parasites. The complex disease in *SOCS1*$^{-/-}$ mice was prevented by administration of anti-IFNγ antibodies and did not occur in *SOCS1*$^{-/-}$ mice also lacking the *IFN*γ gene. Although IFNγ is essential for resistance to a variety of infections, the potential toxic action of IFNγ, particularly in neonatal mice, appears to require regulation. Our data indicate that SOCS1 is a key modulator of IFNγ action, allowing the protective effects of this cytokine to occur without the risk of associated pathological responses.

Introduction

Resistance to infections is dependent on the coordinated action of the cytokine network. Key contributions are made by interferons (IFN; Billiau, 1996; Boehm et al., 1997; De Maeyer and De Maeyer-Guignard, 1998), which comprise two broad groups. Type I interferons include the closely related forms of IFNα and a single form of IFNβ, whereas IFNγ is the sole type II interferon (De Maeyer and De Maeyer-Guignard, 1998). The IFNα proteins and IFNβ are produced by many cell types in response to viral infection (De Maeyer and De Maeyer-Guignard, 1998). In contrast, IFNγ is produced exclusively by activated T cells and natural killer (NK) cells (Billiau, 1996; Boehm et al., 1997; De Maeyer and De Maeyer-Guignard, 1998). IFNγ serves to upregulate expression of a wide variety of genes involved in antigen presentation, activation of macrophages, antiviral, and antiproliferative responses (reviewed by Tatake and Zeff, 1993; Boehm et al., 1997).

The major signal transduction pathway initiated by interferons has been elucidated by a series of elegant biochemical and genetic studies (Darnell et al., 1994; Ihle et al., 1994; Schindler and Darnell, 1995; Bach et al., 1997; Darnell, 1997; Leonard and O'Shea, 1998). IFNγ acts by binding to and inducing the multimerization of a cell surface receptor composed of the IFNGR1 and IFNGR2 chains (Hemmi et al., 1994; Novick et al., 1994). This results in juxtaposition of janus kinase 1 (JAK1) bound to IFNGR1 and JAK2 bound to IFNGR2, which cross-phosphorylate and activate each other (Muller et al., 1993a; Watling et al., 1993; Igarashi et al., 1994). Activated JAKs phosphorylate tyrosine residues within the cytoplasmic domains of the receptor subunits, which act as docking sites for signal transducer and activator of transcription 1 (STAT1; Greenlund et al., 1994; Heim et al., 1995). Phosphorylation of a C-terminal tyrosine (Y701) in STAT1 facilitates interaction with the SH2 domain of a second STAT1 molecule, mediating dimerization (Shuai et al., 1994). STAT1 dimers subsequently migrate to the nucleus, where they bind to gamma-activated sequence (GAS) elements contained within the promoters of IFNγ-inducible genes (Shuai et al., 1992; Muller et al., 1993b), including the genes for inducible nitric oxide synthase (iNOS) and the transcription factor interferon regulatory factor 1 (IRF1). The increased susceptibility to *Mycobacteria*, *Leishmania major*, and some viruses in mice or humans harboring mutations in the genes for IFNγ, its receptor, IRF1, or iNOS highlights the importance of this pathway in resistance to infection (Huang et al., 1993; Matsuyama et al., 1993; Kamijo et al., 1994; MacMicking et al., 1995; Durbin et al., 1996; Meraz et al., 1996; Newport et al., 1996; Lu et al., 1998).

The actions of IFNγ are not always beneficial, since infections may elicit a host response of sufficient magnitude to become life threatening, for example, morbidity associated with *Staphylococcus aureus* infection and the hepatotoxicity associated with hepatitis B infection (Billiau and Vandekerckhove, 1991; Ando et al., 1993; Matthys et al., 1995). The potentially toxic effects of IFNγ, including fatty degeneration of the liver, have also been demonstrated in experiments in which circulating IFNγ levels are experimentally elevated in neonatal mice (Gresser, 1982; Toyonaga et al., 1994). Regulatory mechanisms must therefore exist to maintain the fine balance between beneficial and detrimental responses to IFNγ. This is achieved in part through the production of interleukin-4 (IL-4), IL-10, and IL-13, which counteract the effects of IFNγ (Paul, 1991; Moore et al., 1993; Zurawski and de Vries, 1994), and by negative regulation of IFNγ signal transduction, for example, by the SH2 domain-containing phosphatase SHP1 (Massa and Wu, 1996).

In vitro studies have also implicated a family of SH2-containing proteins, the suppressors of cytokine signaling (SOCS) proteins, in the negative regulation of cytokine signal transduction. Of the eight SOCS proteins (SOCS1 to 7 and CIS), SOCS1 and SOCS3 appear to be

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the most potent inhibitors of cytokine signaling (Nicholson et al., 1999). SOCS1 was initially identified in a functional screen for proteins capable of inhibiting IL-6 signaling (Starr et al., 1997). Subsequent studies have shown that STAT activation in response to many cytokines results in increased transcription of the *SOCS1* and *SOCS3* genes and that, when overexpressed, SOCS1 and SOCS3 inhibit the biological effect of cytokines, including IFNγ, that act through the JAK/STAT pathway (Wang et al., 1996; Endo et al., 1997; Matsumoto et al., 1997; Naka et al., 1997; Starr et al., 1997; Adams et al., 1998; Auernhammer et al., 1998; Bjorbaek et al., 1998; Hilton et al., 1998; Sakamoto et al., 1998; Song and Shuai, 1998; Ito et al., 1999). Consistent with the observation that SOCS1 can inhibit the action of a broad range of cytokines, SOCS1 appears to bind to all four members of the JAK family and inhibit their catalytic activity (Endo et al., 1997; Naka et al., 1997; Nicholson et al., 1999). These studies have led to the view that SOCS1 may be part of a general negative feedback loop regulating cytokine action.

The importance of this regulatory pathway was revealed in mice lacking a functional *SOCS1* gene (*SOCS1*$^{-/-}$ mice). These mice die between 2 and 3 weeks of age of a disease that involves fatty degeneration and necrosis of the liver, macrophage infiltration of several organs, and multiple hematopoietic abnormalities, including severe lymphopenia (Starr et al., 1998; Metcalf et al., 1999). In this report, we establish that *SOCS1*$^{-/-}$ mice are hypersensitive to IFNγ and that the complex multiorgan disease and premature death that develops in these mice can be prevented by administration of neutralizing anti-IFNγ antibodies and is absent in mice lacking both functional *SOCS1* and *IFN*γ genes. We conclude that SOCS1 is a critical regulator of cellular sensitivity to interferon-γ, balancing the beneficial immunological activities with the fatal neonatal effects of this cytokine.

Results

Aberrant IFNγ Signaling in *SOCS1*$^{-/-}$ Mice

The capacity of SOCS1 to inhibit IFNγ signaling in vitro (Starr et al., 1997; Sakamoto et al., 1998; Song and Shuai, 1998) and the observation that administration of this cytokine to neonatal mice induces pathology similar to that observed in *SOCS1*$^{-/-}$ mice (Gresser et al., 1981) suggested that the *SOCS1*-deficient disease may be mediated by IFNγ. To directly compare the effects of IFNγ administration with *SOCS1*$^{-/-}$ pathology, neonatal C57BL/6 mice were injected daily for 14 days with 3 μg of IFNγ. Like *SOCS1*$^{-/-}$ mice, the injected mice died in the second and third weeks of life, and histological examination showed that in addition to hepatic changes, their lungs, heart, and pancreas were infiltrated with macrophages. Moreover, the hematopoietic abnormalities observed, including severe lymphopenia and moderate granulocytosis, were also similar to those seen in *SOCS1*$^{-/-}$ mice (data not shown).

To determine more directly whether IFNγ plays a role in the development of pathology in *SOCS1*$^{-/-}$ mice, we examined them for the presence of a dysregulated signal transduction response to IFNγ. Using an oligonucleotide probe capable of binding to STAT1 and STAT3 in electromobility shift assays, activation of STAT1, a key step in IFNγ signal transduction, was readily detected in the livers of 14-day-old *SOCS1*$^{-/-}$ mice but not *SOCS1*$^{+/-}$ or wild-type mice (Figure 1A). We found, however, no evidence of STAT3 activation (Figure 1A; data not shown), suggesting that dysregulated signaling mediated by other inflammatory cytokines such as leukemia inhibitory factor (LIF), IL-6, and oncostatin M (OSM) that utilize STAT3 was not occurring in *SOCS1*$^{-/-}$ animals.

In addition, expression of mRNA for the IFNγ-inducible genes *IRF1* and *iNOS* was elevated in tissues of *SOCS1*$^{-/-}$ mice (Figure 1B). Likewise, class I MHC expression was markedly elevated in the liver (Figure 1C) and in hematopoietic cells, including thymic and splenic T cells, bone marrow and splenic B cells, and monocytes of *SOCS1*$^{-/-}$ mice (Figure 1D and data not shown). Elevation of class I MHC expression in the thymus, bone marrow, and spleen cells was observed at birth, prior to the development of overt disease.

IFNγ Is Essential for Disease Development and Premature Death in Neonatal *SOCS1*$^{-/-}$ Mice

To directly assess the role of IFNγ in the onset and development of disease in *SOCS1*$^{-/-}$ mice, three litters of mice (29 in total) from *SOCS1*$^{+/-}$ parents were injected twice weekly from birth with a neutralizing anti-IFNγ antibody. Only one death occurred (at day 1, of a *SOCS1*$^{+/-}$ mouse), while the remaining 28 mice remained in good health and were of normal body weight when analyzed at 3 weeks of age (Table 1). Genotyping subsequently revealed that six of these mice were homozygous *SOCS1* mutants, indicating that IFNγ was absolutely required for the premature death of *SOCS1*$^{-/-}$ mice (Figures 2A and 2B).

At 21 days, anti-IFNγ-treated *SOCS1*$^{-/-}$ mice were compared with moribund 12- to 21-day-old *SOCS1*$^{-/-}$ mice and 12- to 21-day-old *SOCS1*$^{+/+}$ and *SOCS1*$^{+/-}$ mice. Untreated *SOCS1*$^{-/-}$ mice showed a uniform series of pathological changes (Figure 3; Tables 1 and 2), including fatty degeneration and necrosis of liver cells, infiltration of the liver, lungs, pancreas, heart, and skin with macrophages, and thickening of the skin epithelium with keratinization. Untreated *SOCS1*$^{-/-}$ mice also exhibited profound alterations in hematopoiesis and lymphopoiesis with atrophy of the thymic cortex, failure of lymphoid follicle development in the spleen, and bone marrow lymphopenia (Figures 4A and 4B; Tables 1 and 2). Remarkably, most organs from anti-IFNγ antibody–treated *SOCS1*$^{-/-}$ mice were normal, the exceptions being minor cuffing of lung vessels and persistence of erythropoiesis in the spleen (Figure 3; Tables 1 and 2). Similarly, hematopoietic abnormalities, including lymphopenia, were also reduced, although not entirely eliminated (Figure 4; Tables 1 and 2). Protection from disease was specifically associated with inhibition of IFNγ because injection of *SOCS1*$^{-/-}$ mice with either anti-IL-6 or control rat immunoglobulin did not alter the kinetics of onset or typical multiorgan nature of the disease (Figure 2B).

Because treatment of *SOCS1*$^{-/-}$ mice with anti-IFNγ antibody did not entirely eliminate disease, we wished to determine whether the residual pathology was due to the involvement of other cytokines or to the inefficient

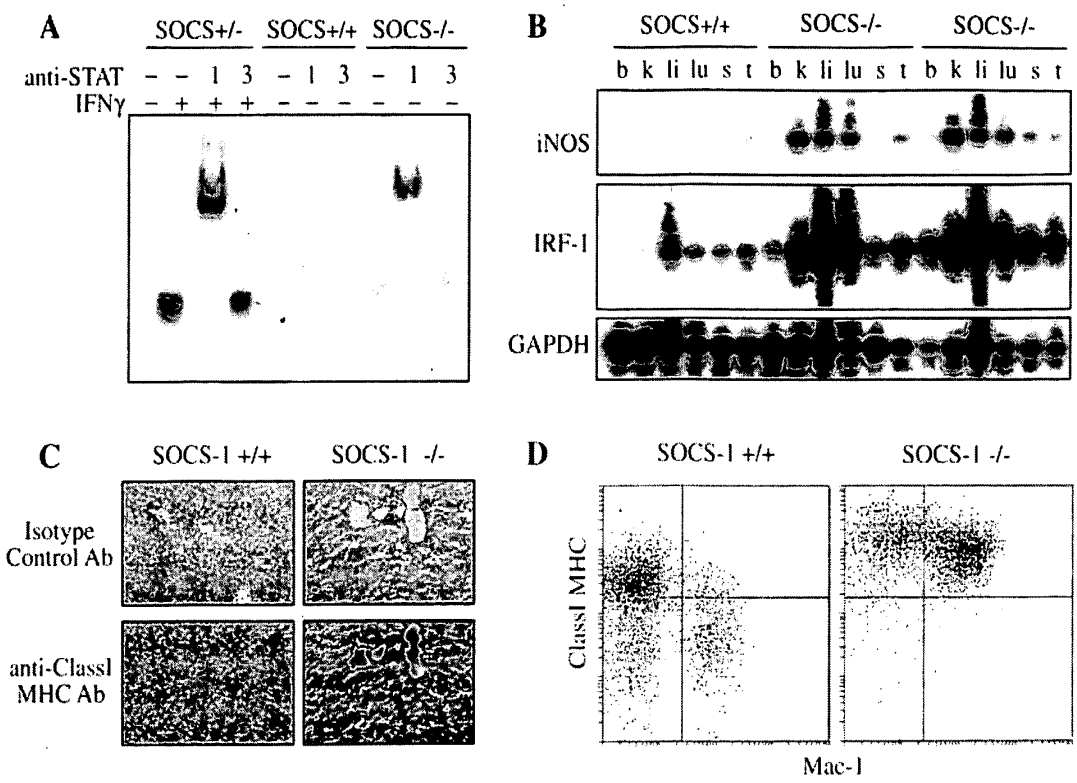

Figure 1. Experimentally Unmanipulated *SOCS1*$^{-/-}$ Mice Show Evidence of an Ongoing Response to IFNγ

(A) EMSA of extracts of livers from *SOCS1*$^{+/-}$ mice injected with 2 μg of IFNγ or untreated *SOCS1*$^{+/+}$ and *SOCS1*$^{-/-}$ mice. Prior to DNA binding, samples were treated, as indicated, with antibodies to either STAT1 or STAT3.

(B) Northern blot analyses showing mRNA from brain (b), kidney (k), liver (li), lung (lu), spleen (s), and thymus (t) from *SOCS1*$^{+/+}$ and *SOCS1*$^{-/-}$ mice hybridized with an *iNOS* probe (upper panel), *IRF1* probe (central panel), or *GAPDH* probe (lower panel).

(C) Immunohistochemistry showing class I MHC expression in the liver. Liver sections were stained with anti-class I MHC antibody or isotype control antibody and a peroxidase-labeled secondary antibody. Sections were counterstained with Giemsa.

(D) FACS analysis of class I MHC and Mac1 expression on bone marrow cells from SOCS1$^{+/+}$ and SOCS1$^{-/-}$ mice. Cells were stained with a biotinylated anti-class I MHC antibody and streptavidin-phycoerythrin followed by an FITC-conjugated anti-Mac1 antibody and were analyzed by flow cytometry. Data shown are representative of at least three independent experiments.

neutralization of IFNγ. To distinguish between these possibilities, we interbred *SOCS1*$^{+/-}$ and *IFNγ*$^{-/-}$ mice to yield animals lacking both SOCS1 and IFNγ. As previous studies of *IFNγ*$^{-/-}$ mice have reported no postnatal death (Dalton et al., 1993), it was not surprising to observe that all *SOCS1*$^{+/+}$ and *SOCS1*$^{+/-}$ mice remained healthy irrespective of their *IFNγ* genotype (data not shown). All *SOCS1*$^{-/-}$ *IFNγ*$^{+/+}$ mice died or became moribund in the second and third weeks of life (Figure 2D), and their disease was identical to that of unmanipulated *SOCS1*$^{-/-}$ mice (Figures 3 and 4; Tables 1 and 2). Consistent with the antibody administration studies, all *SOCS1*$^{-/-}$ *IFNγ*$^{-/-}$ mice survived normally to weaning age and appeared overtly healthy (Figure 2D). A detailed analysis of 12 *SOCS1*$^{-/-}$ *IFNγ*$^{-/-}$ mice at 3 weeks of age revealed normal body and organ weight and no hematological abnormalities (Figure 4D and Table 1). Only 2 of 12 mice showed lymphoid cuffing of lung vessels, and 8 of 12 mice had thymi with an enlarged medulla but normal cortex (Table 2), neither of which were histological features of the typical *SOCS1*$^{-/-}$ disease. *SOCS1*$^{-/-}$ *IFNγ*$^{-/-}$ mice, some of which are now over 6 months old, have remained uniformly healthy, and both males and females have proven fertile. Interestingly, 8 out of 12 *SOCS1*$^{-/-}$ *IFNγ*$^{+/-}$ mice became moribund between 2 and 12 weeks of age (Figure 2D), while the remaining 4 mice were alive after 15 weeks and wer fertile and healthy.

Together, these data from IFNγ antibody treatment and intercrosses with *IFNγ*$^{-/-}$ mice indicate that the full spectrum of disease that results in the postnatal death of *SOCS1*$^{-/-}$ mice is dependent on the action of IFNγ.

SOCS1 Deficiency Results in IFNγ Hyperresponsiveness

The disease in *SOCS1*$^{-/-}$ mice could reflect either an intrinsic cellular hypersensitivity to IFNγ and/or the production of elevated amounts of the cytokine. In this regard, *SOCS1*$^{-/-}$ granulocyte-macrophage progenitor cells have been shown to be hypersusceptible to inhibition by IFNγ (Metcalf et al., 1999). To further investigat these possibilities, the capacity of macrophages grown from bone marrow of wild-type or *SOCS1*$^{-/-}$ mice to kill the intracellular parasite *Leishmania major* following IFNγ stimulation was determined. Macrophages were infected 6 hr after stimulation with various doses of IFNγ

Table 1. Influence of IFNγ on the Development of Hematopoietic Changes in *SOCS1*$^{-/-}$ Mice

SOCS1 genotype	*SOCS1*$^{+/+}$ & *SOCS1*$^{+/-}$	*SOCS1*$^{-/-}$	*SOCS1*$^{+/+}$ & *SOCS1*$^{+/-}$	*SOCS1*$^{-/-}$	*SOCS1*$^{+/+}$ & *SOCS1*$^{+/-}$	*SOCS1*$^{-/-}$
Interferon γ genotype	*IFNγ*$^{+/+}$	*IFNγ*$^{+/+}$	*IFNγ*$^{+/+}$	*IFNγ*$^{+/+}$	*IFNγ*$^{-/-}$	*IFNγ*$^{-/-}$
Treatment	Untreated	Untreated	Anti-IFNγ Ab	Anti-IFNγ Ab	Untreated	Untreated
Number of mice analyzed	10	11	15	4	11	12
Age (days)	12–21	12–21	21	21	21	21
Total peripheral blood white cells/μl	3610 ± 1780	2160 ± 1190	4820 ± 2620	4660 ± 2620	3330 ± 1890	4780 ± 1430
Peripheral blood cells/μl						
Neutrophils	260 ± 100	1130 ± 710	560 ± 300	1890 ± 520	310 ± 150	460 ± 230
Lymphocytes	2850 ± 1580	620 ± 380	3580 ± 1680	1940 ± 1480	2640 ± 1620	3770 ± 1270
Monocytes	370 ± 210	360 ± 220	560 ± 290	440 ± 370	290 ± 220	390 ± 150
Eosinophils	140 ± 100	50 ± 80	70 ± 90	390 ± 320	90 ± 110	160 ± 120
Platelets × 10^{-3}	978 ± 138	630 ± 296	694 ± 195	862 ± 238	748 ± 160	770 ± 180
Hematocrit (%)	33 ± 2	30 ± 3	39 ± 3	36 ± 4	39 ± 3	39 ± 4
Total cells per femur × 10^{-6}	17.5 ± 4.1	5.5 ± 2.1	28.7 ± 9.5	21.6 ± 1.9	21.1 ± 3.0	23.7 ± 3.1
Morphology of bone marrow cells (%)						
Blasts	5 ± 2	6 ± 2	4 ± 2	4 ± 2	4 ± 1	4 ± 1
Myeloblasts/myelocytes	3 ± 2	10 ± 3	5 ± 2	7 ± 1	4 ± 3	3 ± 2
Metamyelocytes/neutrophils	15 ± 5	30 ± 12	17 ± 4	20 ± 7	14 ± 4	15 ± 5
Lymphocytes	38 ± 8	6 ± 4	37 ± 16	30 ± 11	38 ± 6	38 ± 5
Monocytes	8 ± 3	10 ± 4	4 ± 2	4 ± 2	6 ± 2	5 ± 2
Eosinophils	3 ± 2	4 ± 4	3 ± 3	12 ± 3	2 ± 2	4 ± 2
Nucleated erythroid cells	28 ± 6	34 ± 15	30 ± 11	24 ± 15	31 ± 6	31 ± 6
Spleen weight (mg)	41 ± 10	21 ± 8	57 ± 9	90 ± 32	67 ± 16	67 ± 26
Morphology of spleen cells (%)						
Blasts	3 ± 2	3 ± 2	4 ± 2	6 ± 1	2 ± 2	3 ± 2
Myeloblasts/myelocytes	0.4 ± 0.7	3 ± 4	0.2 ± 0.5	0.6 ± 1.1	0.2 ± 0.4	0.4 ± 0.7
Metamyelocytes/neutrophils	2 ± 2	6 ± 4	4 ± 2	2 ± 3	1 ± 1	1 ± 1
Lymphocytes	53 ± 12	8 ± 5	84 ± 4	53 ± 5	47 ± 17	51 ± 18
Monocytes	2 ± 2	6 ± 4	2 ± 1	1 ± 1	1 ± 2	2 ± 2
Eosinophils	2 ± 1	2 ± 3	0.6 ± 0.9	2 ± 1	0.5 ± 0.5	2 ± 1
Nucleated erythroid cells	38 ± 13	72 ± 18	5 ± 3	35 ± 5	48 ± 17	41 ± 19
Thymus weight (mg)	69 ± 11	8 ± 4	71 ± 14	52 ± 12	82 ± 16	83 ± 14
Body weight (g)	8.9 ± 0.7	4.2 ± 0.8	10.2 ± 1.0	9.3 ± 1.7	10.7 ± 0.9	10.6 ± 1.2

Data represent mean values ± standard deviations. The data should be read by pairwise comparison of column 1 versus column 2, column 3 versus column 4, and column 5 versus column 6. Note that the mice in columns 1 through 4 were generated by crossing *SOCS1*$^{-/+}$ mice on an otherwise wild-type background and are therefore all IFNγ$^{+/+}$, whereas the mice in columns 5 and 6 were generated by crossing *SOCS1*$^{-/+}$ *IFNγ*$^{-/+}$ mice or *SOCS1*$^{-/-}$ *IFNγ*$^{-/-}$ mice, and their identity was established by genotyping.

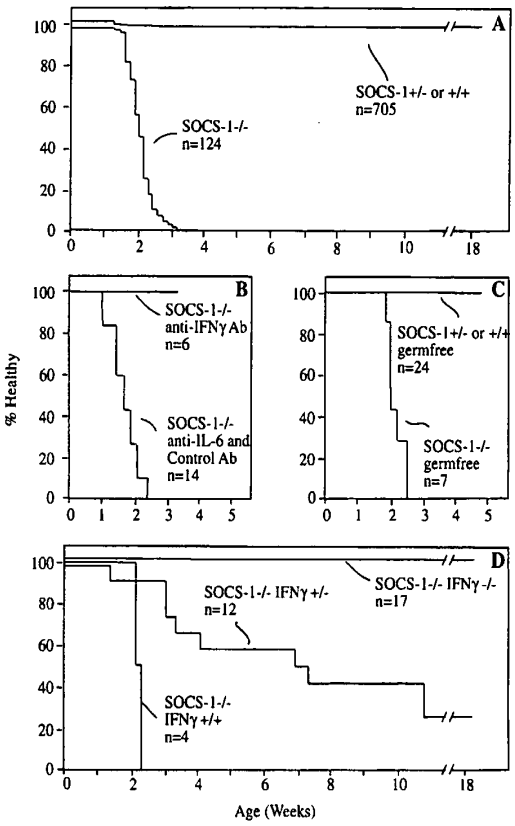

Figure 2. Development of Disease in *SOCS1*$^{-/-}$ Mice Is Dependent on IFNγ and Occurs in a Germ-Free Environment

(A) Rapid onset of morbidity of untreated *SOCS1*$^{-/-}$ but not *SOCS1*$^{+/+}$ or *SOCS1*$^{+/-}$ mice.

(B) Twice weekly injection of neonatal *SOCS1*$^{-/-}$ mice with anti-IFNγ but not anti-IL-6 or control antibody prevents morbidity.

(C) Rapid onset of morbidity in *SOCS1*$^{-/-}$ but not *SOCS1*$^{+/+}$ or *SOCS1*$^{+/-}$ mice born and reared in a germ-free environment.

(D) Prevention of morbidity in *SOCS1*$^{-/-}$ *IFNγ*$^{-/-}$ mice and amelioration of morbidity in *SOCS1*$^{-/-}$ *IFNγ*$^{+/-}$ mice in comparison with *SOCS1*$^{-/-}$ *IFNγ*$^{+/+}$ mice. Seventeen *SOCS1*$^{-/-}$ *IFNγ*$^{-/-}$ mice have been examined to date, with twelve animals being sacrificed for detailed histological and hematological analysis at 3 weeks of age and the remaining five being left for long-term observation.

in the presence of 100 ng/ml lipopolysaccharide. After 2 hr at all IFNγ concentrations, 30% to 50% of macrophages from both *SOCS1*$^{+/+}$ and *SOCS1*$^{-/-}$ mice were infected, and this percentage persisted for up to 48 hr in macrophages of both genotypes in the absence of cytokine (Figure 5A). The ability of IFNγ activation to enhance macrophage killing of *L. major* was demonstrable, because only 1% to 3% of cells from both *SOCS1*$^{+/+}$ and *SOCS1*$^{-/-}$ mice were still infected with live parasites 48 hr after treatment with 10 U/ml of IFNγ (Figure 5A). Strikingly, although ≤0.1 U/ml IFNγ had little or no effect on killing by *SOCS1*$^{+/+}$ macrophages, cells from *SOCS1*$^{-/-}$ mice maintained efficient killing when stimulated with as little as 0.01 U/ml IFNγ (Figure 5A), thus exhibiting at least 100-fold increased sensitivity to IFNγ. The hypersensitivity of macrophages from *SOCS1*$^{-/-}$ mice to IFNγ was confirmed by the demonstration of an increased capacity to produce nitric oxide, the major effector involved in the intracellular killing f *L. major* (data not shown).

The hyperresponsiveness of cells from *SOCS1*$^{-/-}$ mice to IFNγ in vitro was paralleled by an increased resistance of *SOCS1*$^{-/-}$ mice to viral infection in vivo. When 5-day-old mice were infected with Semliki forest virus, all *SOCS1*$^{+/+}$ and *SOCS1*$^{+/-}$ mice died within 2 to 3 days. However, *SOCS1*$^{-/-}$ mice largely survived this early postinfection period and only became ill 3 to 21 days after viral challenge (Figure 5B). This suggests an almost complete resistance to infection, because the survival pattern of infected and uninfected *SOCS1*$^{-/-}$ mice was similar.

IFNγ levels in the serum and in media conditioned by organs taken from *SOCS1*$^{-/-}$ or control littermate mice were investigated using ELISA and/or antiviral assays. At a limit of detection of 3 U/ml, we were unable to detect IFNγ in serum of mice of either genotype, and media conditioned by organs from *SOCS1*$^{-/-}$ mice did not reproducibly contain more IFNγ than those from their wild-type littermates.

Neonatal Death of *SOCS1*$^{-/-}$ Mice Occurs under Germ-Free Conditions

Because IFNγ was clearly central to the development of disease in *SOCS1*$^{-/-}$ mice, we were interested to determine whether subclinical infection in conventional mice might induce increased production of IFNγ and precipitate the early neonatal death of *SOCS1*$^{-/-}$ mice. Three litters of pups produced from the mating of *SOCS1*$^{+/-}$ mice were delivered by cesarean section into a strictly germ-free environment. As expected, *SOCS1*$^{+/+}$ and *SOCS1*$^{+/-}$ pups were among the progeny, and these were weaned normally and as adults exhibited enlarged ceca characteristic of mice in a germ-free environment. In contrast, germ-free *SOCS1*$^{-/-}$ pups died during the second and third weeks of life, the same age at which conventional *SOCS1*$^{-/-}$ mice die (Figure 2C). Analyses of seven of these moribund germ-free *SOCS1*$^{-/-}$ mice revealed that their organ pathology and peripheral blood abnormalities were identical to *SOCS1*$^{-/-}$ mice raised under conventional conditions.

Discussion

SOCS1 Is a Critical Regulator of IFNγ-Mediated Signal Transduction

SOCS1 is indispensable for survival beyond the postnatal period. Mice lacking this regulator die before weaning from a complex disease characterized by fatty degeneration of the liver, macrophage infiltration of several organs, and severe lymphopenia (Starr et al., 1998). We demonstrate here that this fatal syndrome is associated with the specific activation of STAT1 and elevated expression of IRF1, iNOS, and class I MHC, strongly implying that IFNγ signaling pathways are aberrantly active in vivo in the absence of SOCS1. Remarkably, all organ pathology was eliminated or markedly reduced, and death was prevented by treatment of neonatal *SOCS1*$^{-/-}$ mice with a neutralizing anti-IFNγ antibody. The amelioration f disease was specific to the inhibition of IFNγ because treatment of *SOCS1*$^{-/-}$ mice with neutralizing anti-IL-6 antibodies or control immunoglobulin had no

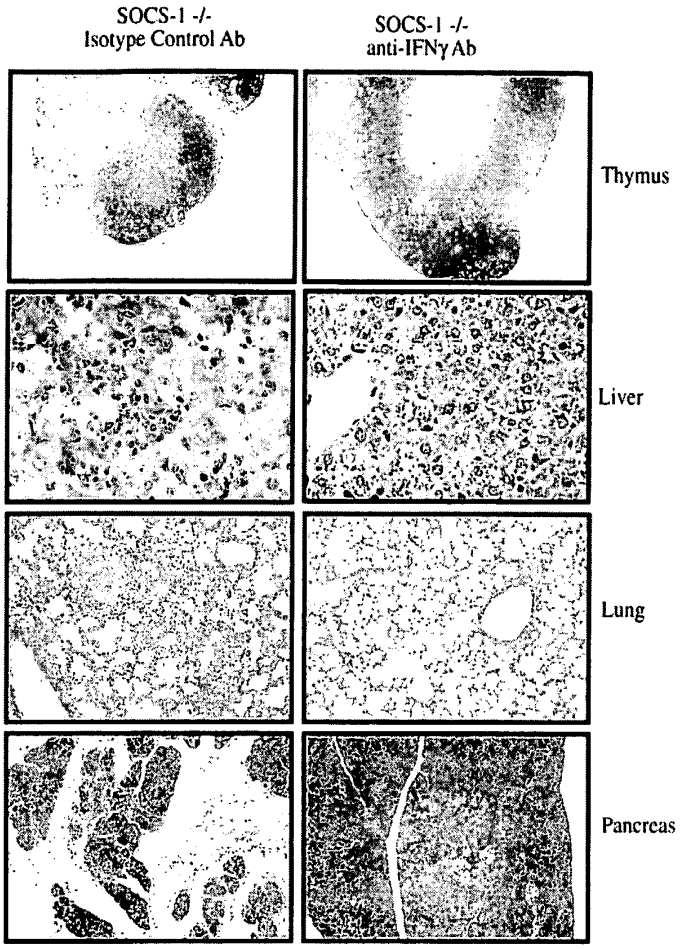

Figure 3. Prevention of Tissue Pathology in *SOCS1*$^{-/-}$ Mice by Treatment with Anti-IFNγ Antibody

Neonatal *SOCS1*$^{-/-}$ mice were treated twice weekly with either anti-IFNγ antibody or control antibody. Anti-IFNγ antibody–treated mice were healthy at 21 days of age and were sacrificed. Control antibody–treated mice developed disease between 9 and 16 days of age and were sacrificed when moribund. The normal histological appearance of organs from anti-IFNγ antibody-treated *SOCS1*$^{-/-}$ mice (right-hand panels) is contrasted with the atrophy of the cortex of the thymus, the fatty degeneration, hematopoietic infiltration and necrosis of the liver, macrophage accumulation around the bronchi and in the alveolar walls in the lung, and macrophage infiltration and acinar tissue destruction of the pancreas observed in *SOCS1*$^{-/-}$ mice treated with control antibody (left-hand panels).

protective effect. We were also unable to find any evidence of dysregulated activation of STAT3 in *SOCS1*$^{-/-}$ mice, the STAT protein used preferentially by inflammatory cytokines such as IL-6 and LIF (Ihle et al., 1994). In addition, the full spectrum of disease development was completely prevented in mice genetically deficient in *IFNγ* as well as *SOCS1*, and a delayed disease developed in *SOCS1*$^{-/-}$ mice that had only a single functional copy of the *IFNγ* gene. The absence of *SOCS1* correlated with marked in vitro hypersensitivity to IFNγ in macrophages infected with the intracellular parasite *L. major*. Hypersensitivity to IFNγ was also observed when nitric oxide (NO) production was measured following stimulation of macrophages with LPS and IFNγ. Likewise, hematopoietic cells from *SOCS1*$^{-/-}$ mice have also been found to be more susceptible to inhibition of proliferation by IFNγ but normally responsive to most hematopoietic cytokines that also act through the JAK/STAT pathway (Metcalf et al., 1999). Together these data clearly establish that SOCS1 is an essential physiological regulator of cellular sensitivity to IFNγ signaling.

Our results do not exclude the possibility that an elevated production of IFNγ in *SOCS1*$^{-/-}$ mice might contribute to disease development. However, within the sensitivity of the assays used, as in normal animals, we were unable to detect IFNγ in the circulation of *SOCS1*$^{-/-}$ mice. Media conditioned by organs of *SOCS1*$^{-/-}$ or normal mice also displayed no consistent differences in IFNγ concentrations. Finally, while *SOCS1*$^{-/-}$ *IFNγ*$^{-/-}$ mice have remained disease free, some *SOCS1*$^{-/-}$ *IFNγ*$^{+/-}$ mice do die with a more slowly developing disease. The delayed disease in mice containing only a single functional *IFNγ* allele suggests that IFNγ concentrations may be limiting in these mice and therefore unlikely to be grossly elevated in *SOCS1*$^{-/-}$ mice. IFNγ is thought to be usually produced by T cells and NK cells in response to infection. Since *SOCS1*$^{-/-}$ mice succumb to disease in strictly germ-free conditions, the IFNγ required for disease development in *SOCS1*$^{-/-}$ mice might either represent basal production or be produced aberrantly. While our studies have clearly demonstrated an indispensable role for IFNγ in the multiorgan pathology of *SOCS1*$^{-/-}$ mice, the source of this cytokine and the identity of potential downstream effectors of IFNγ action remain to be examined.

Sp cificity of SOCS1 Action In Vivo

Previous in vitro data suggested that SOCS1 can directly interact with all four members of the JAK family, resulting in the inhibition of their catalytic activity (Endo et al., 1997; Naka et al., 1997). Consistent with this range of action, overexpression of SOCS1 in cell lines inhibited signal transduction from a large number of cytokines,

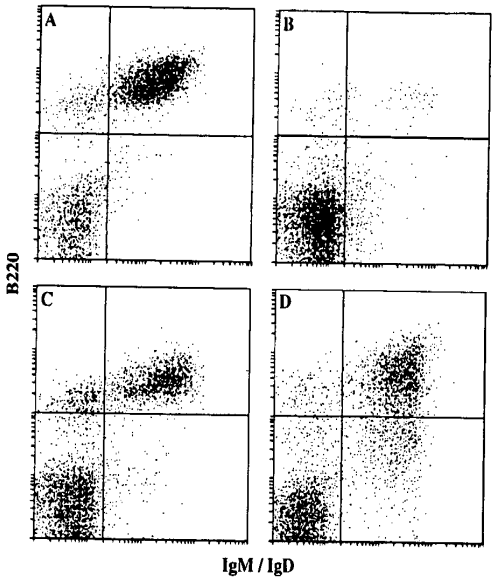

Figure 4. Prevention of B Lymphopenia in *SOCS1*$^{-/-}$ Mice by Treatment with Anti-IFNγ Antibody or Generation of *SOCS1*$^{-/-}$ *IFNγ*$^{-/-}$ Mice

Spleen cells were collected from (A) wild-type mice, (B) *SOCS1*$^{-/-}$ mice treated with a control antibody, (C) *SOCS1*$^{-/-}$ mice treated with anti-IFNγ antibody, and (D) *SOCS1*$^{-/-}$ *IFNγ*$^{-/-}$ mice. Cells were stained with anti-B220 antibody and a mixture of anti-IgM and anti-IgD antibodies and analyzed by FACS.

including IFNα, IFNβ, IFNγ, IL-6, LIF, OSM, thrombopoietin, growth hormone, and stem cell factor (SCF). Moreover, each of these cytokines is able to rapidly induce the synthesis of *SOCS1* mRNA (Endo et al., 1997; Starr et al., 1997; Adams et al., 1998; Auernhammer et al., 1998; Bjorbaek et al., 1998; Sakamoto et al., 1998; Song and Shuai, 1998; Ito et al., 1999; De Sepulveda et al., 1999).

Given the promiscuity of SOCS1 action in vitro, disease in mice lacking this protein might potentially have been due to deregulation of signals from a multitude of cytokines. Remarkably, however, the full spectrum of the complex disease in *SOCS1*$^{-/-}$ mice, including liver degeneration, hematopoietic infiltration of multiple tissues, and profound loss of T and B lymphocytes, can be attributed to a failure to regulate signal transduction by a single cytokine, IFNγ. This implies that in contrast to its actions in vitro, under physiological conditions SOCS1 has no indispensable role in the regulation of signal transduction from the majority of cytokines that utilize the JAK-STAT pathway.

As the in vitro studies all involved overexpression of SOCS1, it is possible that these approaches overestimated the breadth of SOCS action, simply because the level of *SOCS* gene expression achieved experimentally is likely to be far higher than expression controlled by an endogenous promoter. Moreover, ectopic expression may also be temporally inappropriate because physiological transcription of *SOCS* genes appears to be dependent on exposure to cytokine, allowing SOCS proteins to act in vivo only after signaling has commenced. However, although it is clear that a lack of SOCS1 causes a lethal hyperresponsiveness to IFNγ, abnormal responses to other cytokines may not be manifested so dramatically. Our studies to date suggest that responses to most hematopoietic cytokines are not altered in *SOCS1*$^{-/-}$ mice (Metcalf et al., 1999). However, the postnatal lethality in mice lacking this protein has prevented analysis of signaling by these or other cytokines in the adult mouse. Clearly, the availability of healthy *SOCS1*$^{-/-}$ *IFNγ*$^{-/-}$ mice will now allow the full spectrum of in vivo SOCS1 activity to be definitively resolved.

Binding of IFNγ to its cell surface receptor triggers the activation of JAK1 and JAK2. While in vitro studies have shown SOCS1 is able to inhibit the function of both these kinases, the biochemical specificity of SOCS1 action in vivo remains to be determined. The inhibition of JAK activity by SOCS1 may regulate IFNγ signaling in several ways, for example, by determining the threshold concentration of IFNγ capable of triggering a biological response or by regulating the magnitude or length of a response to a particular concentration of IFNγ. Distinguishing between these possibilities will require a careful comparison of the capacity of different doses of IFNγ to induce JAK1 and JAK2 activation, IFNγ receptor phosphorylation, STAT1 activation, and expression of STAT1-regulated genes in wild-type and SOCS1-deficient cells.

As SOCS1 is a member of a larger family of related proteins, it is also feasible that a regulatory role for this protein in other cytokine signaling pathways is not evident in *SOCS1*$^{-/-}$ mice due to the compensatory actions of other SOCS proteins. In vitro studies have clearly shown that SOCS1 and SOCS3 have overlapping activities, and both molecules are induced by and inhibit the actions of a similar spectrum of cytokines when overexpressed (Adams et al., 1998; Sakamoto et al., 1998; Song and Shuai, 1998; Ito et al., 1999). However, for regulation of IFNγ signaling, SOCS1 appears to be considerably more active than SOCS3 (Sakamoto et al., 1998; Song and Shuai, 1998). Consistent with these in vitro studies, the phenotype of *SOCS1*$^{-/-}$ mice reveals key roles for SOCS1 in regulating IFNγ responses that cannot be compensated by SOCS3 in vivo. Whether SOCS3 also acts to regulate a unique set of cytokine signal transduction pathways and whether there are other pathways regulated by both SOCS1 and SOCS3 under physiological conditions remains to be clarified.

SOCS1 Balances the Beneficial and Potentially Deleterious Actions of IFNγ

The potentially devastating actions of IFNγ in vivo have been well established in studies in which IFNγ levels have been experimentally elevated (Gresser et al., 1981, 1987; Gresser, 1982), and these pathologies were faithfully reproduced in mice lacking SOCS1. In addition, inappropriate IFNγ production and action is implicated in the pathogenesis of disease following certain infections. In a mouse transgenic model of hepatitis B, the destruction of liver cells and resultant morbidity appears to be caused by excess endogenous IFNγ (Ando et al., 1993). In other models of infection, however, the actions of IFNγ are beneficial (Huang et al., 1993; Newport et al., 1996; Lu et al., 1998), and this action is also amplified in mice lacking SOCS1. SOCS1-deficient macrophages

Table 2. Influence of IFNγ on the Development of Histological Changes in Organs of *SOCS1*$^{-/-}$ Mice

SOCS1 genotype	*SOCS1*$^{+/+}$ & *SOCS1*$^{+/-}$	*SOCS1*$^{-/-}$	*SOCS1*$^{+/+}$ & *SOCS1*$^{+/-}$	*SOCS1*$^{-/-}$	*SOCS1*$^{+/+}$ & *SOCS1*$^{+/-}$	*SOCS1*$^{-/-}$
Interferon γ genotype	*IFNγ*$^{+/+}$	*IFNγ*$^{+/+}$	*IFNγ*$^{+/+}$	*IFNγ*$^{+/+}$	*IFNγ*$^{-/-}$	*IFNγ*$^{-/-}$
Treatment	Untreated	Untreated	Anti-IFNγ Ab	Anti-IFNγ Ab	Untreated	Untreated
Age (days)	12–21	12–21	21	21	21	21
Liver						
Fatty degeneration	0/21	12/18	0/22	0/6	0/8	0/12
Necrosis	0/21	6/18	0/22	0/6	0/8	0/12
Excess hematopoiesis	0/21	18/18	0/22	0/6	0/8	0/12
Lung						
Cuffing by mϕ and neut	1/21	17/18	0/22	2/6	0/8	2/12
Heart						
Infiltration by mϕ and neut	0/21	15/17	0/19	1/6	0/8	0/12
Kidney						
Glomerular immaturity	2/21	16/18	1/18	0/6	1/8	1/12
Tubular immaturity	6/21	11/18	0/18	0/6	0/8	0/12
Spleen						
Lymphoid follicles	20/21	1/19	21/21	5/5	8/8	12/12
Excess erythropoiesis	1/21	15/17	0/21	4/5	3/8	9/12
Thymus						
Cortical atrophy	0/21	15/16	0/22	1/5	0/8	0/12
Medullary enlargement	1/21	1/16	0/22	1/5	1/8	8/12
Pancreas						
Acinar atrophy	2/19	16/18	0/22	0/5	0/8	0/12
Infiltration by mϕ	0/19	13/18	0/22	0/5	0/8	0/12
Skin						
Mϕ infiltration	0/20	14/17	0/10	1/4	0/8	0/12
Epidermal thickening	0/20	15/17	0/10	0/4	0/8	0/12
Keratinization	0/20	14/17	0/10	0/4	0/8	0/12
Eosinophil infiltration	0/20	6/17	0/10	0/4	0/8	0/12
Marrow						
Monos > granulocytes	16/20	2/18	16/19	3/5	8/8	12/12
Granulocytes > monos	4/20	16/18	3/19	2/5	0/8	0/12

The ratios indicate the number of organs showing a particular lesion versus the total number of organs examined. The data should be read by pairwise comparison of column 1 versus column 2, column 3 versus column 4, and column 5 versus column 6. Note that the mice in columns 1 through 4 were generated by crossing *SOCS1*$^{-/+}$ mice on an otherwise wild-type background and are therefore all *IFNγ*$^{+/+}$, whereas the mice in columns 5 and 6 were generated by crossing *SOCS1*$^{-/+}$ *IFNγ*$^{-/+}$ mice or *SOCS1*$^{-/-}$ *IFNγ*$^{-/-}$ mice, and their identity was confirmed by genotyping. Not all organs were sectioned from all animals. Neut, neutrophil; monos, mononuclear cells.

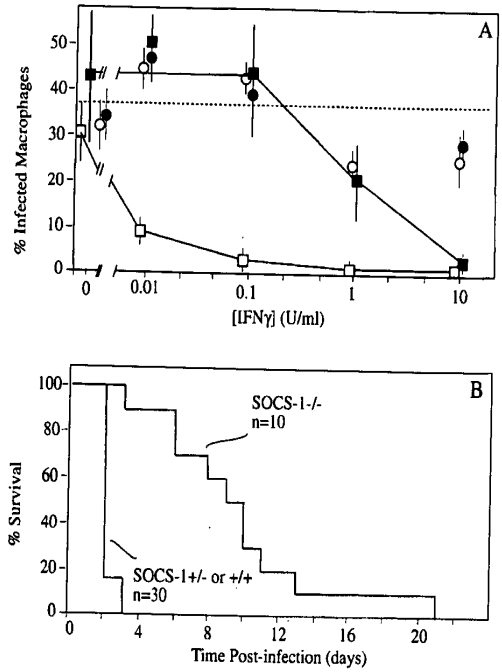

Figure 5. Absence of SOCS1 Renders Cells Hyperresponsive to IFNγ and Mice Hyperresistant to Semliki Forest Virus Infection

(A) Macrophages from the bone marrow of *SOCS1*$^{+/+}$ (filled symbols) and *SOCS1*$^{-/-}$ mice (open symbols) were stimulated with 100 ng/ml LPS and the indicated concentration of IFNγ and infected with *L. major*. After 2 hr (circles) and 48 hr (squares), the percentage of macrophages containing parasites was determined.

(B) Mice generated by crossing *SOCS1*$^{+/-}$ mice were infected with Semliki forest virus at 5 days of age and monitored.

stimulated with IFNγ killed intracellular parasites considerably more efficiently than their wild-type counterparts, and *SOCS1*$^{-/-}$ mice were more resistant than normal mice to Semliki forest virus infection. Clearly, the actions of IFNγ in vivo require exquisite control. The data presented here establish that SOCS1 is central to the balance between the beneficial and potentially harmful effects of IFNγ. While the behavior of neonatal mice may not necessarily predict the occurrence of similar responses to overstimulation by IFNγ in humans, these observations nevertheless raise the prospect that small molecule mimetics or antagonists of SOCS1 might prove clinically valuable in enhancing the actions of IFNγ in combating viral and parasitic infection or in dampening its pathological side effects in other disease states.

Experimental Procedures

Generation and Maintenance of Mice and Injection of Antibodies, Cytokine, and Virus

SOCS1$^{-/-}$ mice were generated as described previously (Starr et al., 1998) and maintained on a mixed 129/Sv and C57BL/6 genetic background. *IFNγ*$^{-/-}$ mice on an inbred C57BL/6 background (C57BL/6-Ifng^{tm1Ts}) were obtained from the Jackson Laboratories via Monash University (Dalton et al., 1993). Mice were routinely housed in clean but not specific pathogen-free conditions (SPF) at the Walter and Eliza Hall Institute of Medical Research. To raise mice under germ-free conditions, pups were delivered by cesarean section and placed with BALB/c foster mothers that had been maintained in germ-free microisolators for several generations. The sterility of this environment was monitored closely and tested continually for the absence of bacterial organisms and a variety of viral pathogens.

Mice were genotyped by Southern blot analysis of genomic DNA obtained from tail tips, as described (Starr et al., 1998). For analysis of the *SOCS1* gene, genomic DNA was digested with EcoRI and filters were probed with a 1.5 kilobase pairs (kbp) EcoRI/HindIII fragment of the mouse *SOCS1* gene, which hybridizes to a 5.3 kbp band for the wild-type allele and an 8.0 kbp fragment for the null allele. For analysis of the *IFNγ* gene, DNA was cut with BamHI and filters were probed with a 450 bp PstI fragment of the mouse *IFNγ* cDNA that detects a fragment of 11.0 kbp for the wild-type allele and 13.0 kbp for the null allele.

Neonatal mice were injected intraperitoneally twice weekly for up to 3 weeks with 16 mg/kg of neutralizing rat anti-IFNγ antibody (R4-6A2; American Type Culture Collection, Manassas, VA), 200 mg/kg of an ammonium sulfate–precipitated neutralizing polyclonal rabbit anti-IL-6 antisera, as described (Liu et al., 1995), 16 mg/kg control purified rat IgG antibody (Sigma Chemical Co., St. Louis, MO), or 200 mg/kg ammonium sulfate–precipitated preimmune rabbit sera and were sacrificed when moribund or at the end of the experiment. The efficacy of anti-IL-6 treatment was verified at the end of the experiment by testing serum from injected mice for its capacity to inhibit IL-6-induced differentiation of M1 cells. In a titration experiment, a 1 in 100 dilution of serum from mice injected with the anti-IL-6 but not control antibody was found to inhibit 60 ng/ml of IL-6.

In some experiments, 5-day-old mice were injected intraperitoneally with 50 μl of Semliki forest virus containing 100 times the virus dose that kills 50% of fibroblasts in vitro. The mice were monitored daily and sacrificed when moribund.

IFNγ Assays

IFNγ in serum was assayed using cytopathic effect reduction with mouse L929 cells as targets for Semliki forest virus challenge as described (Hertzog et al., 1991). A standard cytokine sandwich ELISA was also used to quantitate IFNγ in mouse serum and organ-conditioned media. IFNγ-specific antibodies (R4-6A2 as the capture antibody, XMG1.2 for detection) were obtained from Pharmingen (San Diego, CA), and the assay was performed essentially as described by the manufacturer.

Hematological Analysis, Histology, and Immunohistochemistry

Hematological analyses were performed as described (Starr et al., 1998; Metcalf et al., 1999). For histological examination, tissues were fixed in 10% (v/v) formalin in phosphate-buffered saline (PBS) and sections were prepared and stained by standard techniques (Starr et al., 1998). Tissue preparation for immunohistochemistry was carried out as described (Thomas et al., 1998), and sections were stained with a rat anti-mouse class I MHC antibody, 34-12S, and a peroxidase-labeled secondary antibody. Sections were counterstained with Giemsa (Ozato et al., 1982).

Culture of Macrophages and Leishmanicidal Assays

To obtain bone marrow–derived macrophages, femoral bone marrow cells collected in PBS were dispersed, centrifuged, and resuspended in 10 ml Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 ng/ml murine macrophage colony-stimulating factor (M-CSF; Cetus Corporation, Emeryville, CA) and 10% (v/v) fetal bovine serum (FBS). After 3 days of culture in a humidified incubator at 37°C in 5% (v/v) CO_2 in air, medium and nonadherent cells containing an expanded precursor population were resuspended at 10^4 cells/ml and cultured for an additional 5 days in the presence of M-CSF, after which a relatively pure and homogeneous population of adherent mature bone marrow macrophages were present.

The cloned line of *L. major* LRC-L137 V 121 has been described in detail (Handman, 1983). Promastigotes were grown in M199 medium with 10% FBS and were in the stationary phase of growth. Infection of macrophages was carried out using a modification of a method described previously (Proudfoot et al., 1995). Briefly, 5×10^4 bone marrow–derived macrophages were transferred onto glass coverslips in 24-well trays, allowed to adhere for 24 hr, and stimulated with 100 ng/ml LPS and various concentrations of IFNγ. After 6 hr of cytokine stimulation, cells were infected at a ratio of five parasites

per cell for 2 hr at 37°C. The free parasites were removed by vigorous washing before incubation for a further 24 or 48 hr, after which cells were stained with Giemsa. The percentage of macrophages infected with parasites was determined at each time point, with at least 400 cells per sample counted.

Northern Blots

Following sacrifice, organs were removed from neonatal wild-type and *SOCS1*$^{-/-}$ mice. PolyA$^+$ mRNA was purified, and Northern blot hybridization was performed essentially as described (Alexander et al., 1995). Probes used were as follows: a 1.1 kbp PstI fragment of the chicken *glyceraldehyde 6-phosphate dehydrogenase* (*GAPDH*) cDNA, a 2 kbp EcoRI fragment of the mouse *IRF1* gene, and a 1.8 kbp NcoI fragment of the mouse *iNOS* cDNA.

Flow Cytometry

Single-cell suspensions of femoral bone marrow, thymocytes, and splenocytes were prepared and erythrocytes were lysed by incubation in 156 mM ammonium chloride (pH 7.3) at 37°C for 3 min. The cells were stained with a biotinylated or FITC-conjugated rat monoclonal antibody specific for the cell surface markers of interest (Class I MHC, CD4, CD8, surface IgM, surface IgD, B220, or Mac1), followed where necessary by streptavidin-phycoerythrin and analyzed by flow cytometry as described (Strasser et al., 1991).

Electrophoretic Mobility Shift Assays

Nuclei were extracted from the livers of unmanipulated *SOCS1*$^{-/-}$ mice and control littermates or from mice 15 min after intraperitoneal administration of 2 μg IFNγ (Ruff-Jamison et al., 1993). Electrophoretic mobility shift assays (EMSA) were performed on 1–2 μg of nuclear protein using the m67 oligonucleotide probe as described (Novak et al., 1995).

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Ovine genomic DNA was prepared from cell pellets, tail samples of live lambs and umbilical cord samples of dead lambs. Southern analysis was carried out by standard procedures. Three hybridization probes were used: a 5′ internal probe, a 3-kb *COL1A1* *Sal*I, *Ssp*I fragment corresponding to the 5′ homologous arm of COLT-1 and 2 (Fig. 1); a 5′ external probe, a 520-bp ovine *COL1A1* fragment directly adjacent to but outside the 5′ homologous arm; a 3′ internal probe, a 0.7-kb *COL1A1* *Sal*I, *Pst*I fragment immediately 3′ of the integration site (Fig. 1). The diagnostic fragments detected were: a 4.7-kb *Bam*HI fragment extending across the 5′ junction of the targeted locus from a *Bam*HI site within the IRES–*neo* region to a *Bam*HI site in the *COL1A1* gene 5′ of the region contained in the vector; a 8.4-kb *Kpn*I, *Asp*I fragment extending across the 3′ junction of the targeted locus from a *Kpn*I site within the vector to an *Asp*I site in the *COL1A1* gene flank 3′ of the region contained in the vector.

Hormonal induction of lactation

Milk samples were obtained from immature ewes by hormonal induction of lactation, essentially as described[25].

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Gigantism in mice lacking suppressor of cytokine signalling-2

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Suppressor of cytokine signalling-2 (SOCS-2) is a member of the suppressor of cytokine signalling family, a group of related proteins implicated in the negative regulation of cytokine action through inhibition of the Janus kinase (JAK) signal transducers and activators of transcription (STAT) signal-transduction pathway[1]. Here we use mice unable to express SOCS-2 to examine its function *in vivo*. SOCS-2$^{-/-}$ mice grew significantly larger than their wild-type littermates. Increased body weight became evident after weaning and was associated with significantly increased long bone lengths and the proportionate enlargement of most organs. Characteristics of deregulated growth hormone and insulin-like growth factor-I (IGF-I) signalling, including decreased production of major urinary protein, increased local IGF-I production, and collagen accumulation in the dermis, were observed in SOCS-2-deficient mice, indicating that SOCS-2 may have an essential negative regulatory role in the growth hormone/IGF-I pathway.

We isolated genomic clones corresponding to three independent loci from two murine libraries using a SOCS-2 coding region complementary DNA as hybridization probe. Comparison of sequence from these clones with that of the SOCS-2 cDNA revealed that one locus, which consisted of three exons and two introns, encoded the predicted SOCS-2 RNA (Fig. 1a). The two other loci

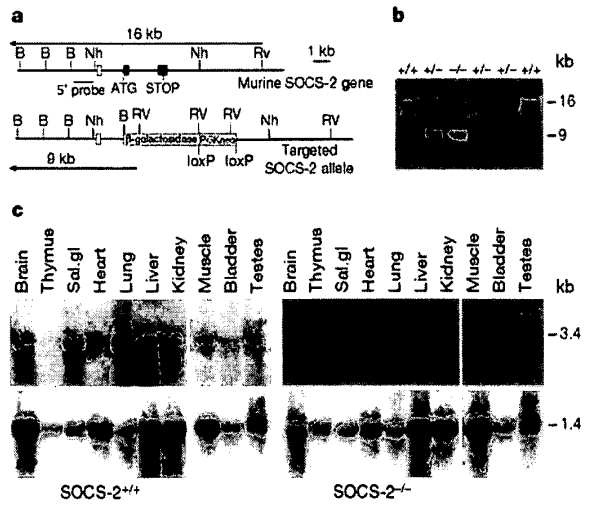

Figure 1 Disruption of the SOCS-2 locus by homologous recombination. **a**, The functional murine SOCS-2 gene (B, *Bam*HI; Nh, *Nhe*I; RV, *Eco*RV) with the exons containing the coding region as shaded boxes. In the targeted allele, the entire SOCS-2 coding region was replaced by a β-gal-PGKneo cassette in which the β-galactosidase coding region was fused to the SOCS-2 initiation codon. **b**, Southern blot of *Eco*RV-digested genomic DNA from the tails of mice derived from a cross between SOCS-2$^{+/-}$ mice. The blot was hybridized with the 5′ genomic SOCS-2 probe, which distinguishes between endogenous (16 kb) and mutant SOCS-2 (9 kb) alleles. **c**, Northern blot showing lack of SOCS-2 expression in organs of SOCS-2$^{-/-}$ mice. Top, the blot was hybridized with a coding region probe, which detects the 3.4-kb SOCS-2 transcript[1]; bottom, the integrity of the RNA was confirmed by hybridization with GAPDH (1.4-kb transcript). Sal gl, salivary gland.

represented processed or rearranged genes. Only one of these additional loci retained an intact SOCS-2 open reading frame, but it lacked the upstream region, exon 1 and part of exon 2. No cDNA clones corresponding to RNAs expressed from these rearranged loci were detected. We constructed a targeting vector for the deletion of the two coding exons in the SOCS-2 gene by homologous recombination in embryonic stem cells (Fig. 1a). Chimaeric mice were generated from an embryonic stem cell clone bearing the targeted locus, and animals lacking one functional SOCS-2 gene were bred. Southern blot analysis at weaning revealed that offspring of heterozygous parents included mice of each of the three expected genotypes (Fig. 1b) in approximately mendelian proportions (22:51:22 for SOCS-$2^{+/+}$:SOCS-$2^{+/-}$:SOCS-$2^{-/-}$). Northern blots of RNA extracted from a range of organs confirmed that SOCS-2 transcripts were absent in homozygous mutant mice (Fig. 1c). This result is consistent with a lack of expression from the alternative SOCS-2 loci and confirmed that the SOCS-2 gene had been functionally deleted.

SOCS-2-deficient mice were indistinguishable from their littermates until weaning at three weeks of age but subsequently grew more rapidly (Figs 2a, 3a). SOCS-$2^{-/-}$ males weighed significantly more than their wild-type counterparts at six weeks of age and as adults were, on average, 40% heavier. Adult SOCS-$2^{+/-}$ males exhibited an intermediate body weight (−/−: 36.6 ± 1.0 g; +/−: 30.8 ± 1.3 g; +/+:27.1 ± 1.6 g, at 12 weeks of age, n = 5–20 mice per group). Increased growth was also significant but less marked in female SOCS-$2^{-/-}$ mice (Fig. 2a). Adult SOCS-$2^{-/-}$ females typically attained the weight of wild-type male mice, but heterozygous SOCS-2 females were not significantly heavier than sex-matched wild-type littermates (−/−: 26.2 ± 1.5 g; +/−: 21.8 ± 0.7 g; +/+: 20.5 ± 1.4 g, at 12 weeks of age, n = 7–20 mice per group).

Visual examination and measurement of abdominal fat mass indicated that neither male nor female SOCS-$2^{-/-}$ mice accumulated significantly more fatty tissue than wild-type mice. Rather, increased body weight in these mice resulted from an increase in the weight of most visceral organs (Fig. 2b) of similar magnitude to the increase in overall body weight. Sexually dimorphic or male-specific organs were not disproportionately enlarged. Carcass weight was also increased, indicating that muscle and bone may contribute significantly to the increased size of SOCS-2-deficient mice. Consistent with this interpretation, the femur, tibia, radius and humerus in SOCS-$2^{-/-}$ mice were all significantly longer than in wild-type controls (Table 1). Body length in male SOCS-2-deficient mice was also greater, although tail length was normal (Table 1). The mean frequency of hepatic nuclei per 10 high-power fields in SOCS-$2^{-/-}$ liver sections was no greater than that in wild-type mice (27.3 ± 4.4, n = 4 versus 27.7 ± 2.6, n = 4), and striated muscle cell width was normal in the thighs of SOCS-2-deficient animals. Thus,

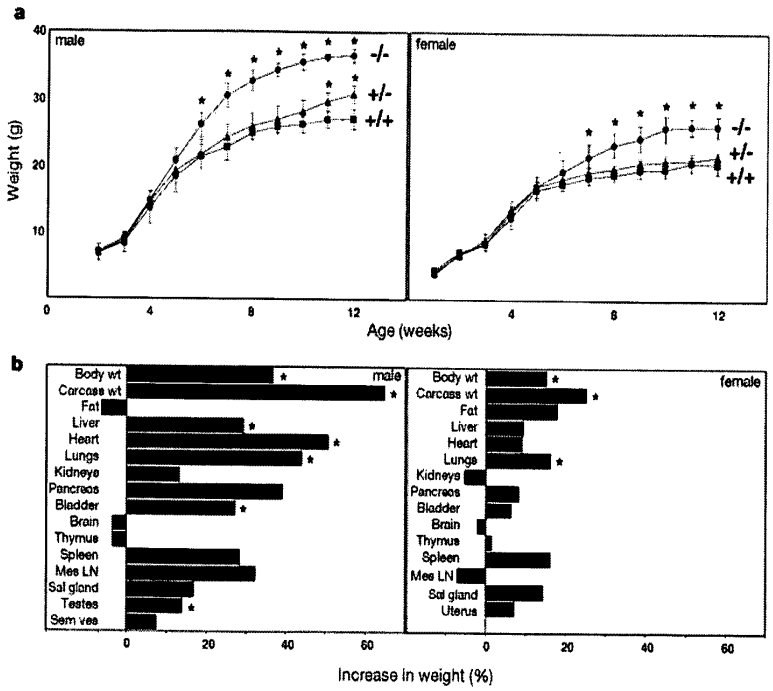

Figure 2 Excessive growth of SOCS-$2^{-/-}$ mice. **a**, Growth curves for male and female SOCS-$2^{+/+}$ (squares), SOCS-$2^{+/-}$ (triangles) and SOCS-$2^{-/-}$ (circles) mice. Body weights from cohorts of mice weighed at weekly intervals are shown: each point represents mean ± s.d. for 5–20 mice. **b**, Percentage increase in body, carcass and organ weights in SOCS-$2^{-/-}$ mice over that determined in age-matched wild-type mice (n = 7–8 three-month-old mice per measurement). Asterisk, measurements from SOCS-$2^{-/-}$ mice that were significantly different from wild-type controls (P < 0.05, Student's t-test). wt, weight; mes LN, mesenteric lymph node; sal gland, salivary gland; sem ves, seminal vesicles.

Table 1 Body, tail and bone lengths in SOCS-$2^{-/-}$ mice

	Length (mm)					
	Male			Female		
	SOCS-$2^{+/+}$	SOCS-$2^{+/-}$	SOCS-$2^{-/-}$	SOCS-$2^{+/+}$	SOCS-$2^{+/-}$	SOCS-$2^{-/-}$
Body	95.0 ± 1.9	98.8 ± 1.6*	107 ± 0.9*	90.1 ± 2.1	91.3 ± 1.2	100.3 ± 2.1*
Tail	88.3 ± 1.5	88.7 ± 2.1	89.2 ± 1.5	84.8 ± 1.4	88.1 ± 1.6	88.9 ± 1.9*
Femur	18.0 ± 0.2	18.7 ± 0.2*	19.5 ± 0.2*	17.3 ± 0.2	17.7 ± 0.2*	18.6 ± 0.3*
Tibia	20.0 ± 0.2	20.6 ± 0.2*	20.9 ± 0.1*	19.5 ± 0.2	19.9 ± 0.2*	20.5 ± 0.3*
Radius	12.0 ± 0.2	12.4 ± 0.2*	12.7 ± 0.2*	11.6 ± 0.2	11.7 ± 0.2	12.0 ± 0.2*
Humerus	13.8 ± 0.2	14.4 ± 0.3*	15.4 ± 0.2*	13.2 ± 0.2	13.7 ± 0.3*	14.6 ± 0.1*

* P < 0.005 in Student's t-test for comparison of sex-matched SOCS-$2^{-/-}$ and SOCS-$2^{+/-}$ data with SOCS-$2^{+/+}$ mice (n = 6–12).

increased organ weights in these mice apparently result from elevated cell numbers rather than increased cell size. A similar but less pronounced trend was also observed when organ and carcass weights and body, tail and bone lengths were assessed in female SOCS-2$^{-/-}$ mice (Fig. 2b and Table 1).

In adult SOCS-2$^{-/-}$ mice, most organs appeared histologically normal, including the kidney, heart, spleen, thymus, lymph nodes, femur, sternum, gonads and bladder. We did, however, observe a marked thickening of the dermis associated with excessive collagen accumulation in all of seven male, and less prominently in six of eight female, SOCS-2$^{-/-}$ mice (Fig. 3b, c). Collagen deposition was also increased around the bronchi and vessels of the lungs of six of nine male, but only three of ten female, SOCS-2-deficient mice; this occasionally also involved some alveolar sacs (Fig. 3d, e). In a minority of SOCS-2-deficient animals of both sexes, excess collagen accumulation was also observed around occasional hepatic vessels and bile ducts, as well as in duct tissue of the salivary glands and pancreas. No abnormalities in bone architecture were evident in histological sections of adult mice, including the epiphiseal growth plates of the femur and tibia.

SOCS-2 expression can be induced by stimulation of haemopoietic tissues with cytokines[1]. However, a survey of 14 SOCS-2$^{-/-}$ mice at 2 months of age did not identify any haematological abnormalities. The haemtocrit and numbers of platelets, lymphocytes, monocytes, neutrophils and eosinophils in the peripheral blood of SOCS-2$^{-/-}$ mice were normal. The spleen weight and cellularity of the femoral bone marrow and the peritoneal cavity were increased in SOCS-2-deficient mice, but only in parallel with their elevated body weight, and the percentages of morphologically identifiable cells in cytocentrifuge preparations of cells from each of these sites were normal. Flow cytometric analysis of cells from bone marrow, spleen and thymus, using monoclonal antibodies specific for a range of T- and B-lymphoid, myeloid and erythroid markers, revealed no consistent perturbations in SOCS-2-deficient mice. Similarly, cultures of bone marrow and spleen cells from five SOCS-2$^{-/-}$ and five wild-type mice showed similar total colony numbers and proportions of colony subtypes when independently stimulated by GM-CSF (granulocyte-macrophage colony-stimulation factor), G-CSF (granulocyte-CSF), M-CSF (macrophage-CSF), interleukin (IL)-3, stem cell factor (SCF), IL-6 or Flk-ligand plus leukaemia inhibitory factor (LIF) (data not shown).

As members of the SOCS family can inhibit signals emanating from cytokine receptors[2], an attractive hypothesis for the excess growth of SOCS-2$^{-/-}$ mice is that SOCS-2 is a negative regulator of signalling from growth-promoting cytokines. Growth hormone is a key regulator of post-natal growth, acting largely through production of IGF-I (refs 3, 4). The accelerated growth of SOCS-2$^{-/-}$ mice, beginning at about 3–4 weeks of age, coincides with the upregulation of tissue growth hormone receptor expression[5,6], and the excess bone growth seen in SOCS-2$^{-/-}$ mice is a specific characteristic of growth hormone transgenic mice that is also observed in humans with elevated growth hormone[7,8]. However, the adult weights and rates of growth in SOCS-2$^{-/-}$ mice more closely resemble those seen in IGF-I transgenic mice[7]. The excess collagen deposition observed in the dermis of SOCS-2$^{-/-}$ mice is a common characteristic of both growth hormone and IGF-I transgenic mice[9,10], and of humans with

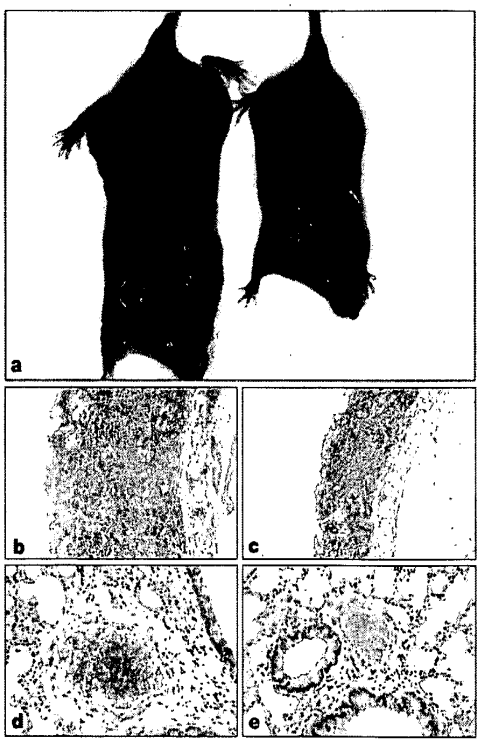

Figure 3 Pathology in SOCS-2$^{-/-}$ mice. **a**, Increase in size of a typical two-month-old male SOCS-2$^{-/-}$ mouse (left) relative to an age- and sex-matched wild-type animal (right). **b**, Van Giessen-stained section of skin from a two-month-old male SOCS-2$^{-/-}$ mouse showing dermis thickened by increased collagen deposits. **c**, Skin of age- and sex-matched wild-type mouse. **d**, Haematoxylin and eosin-stained section showing collagen deposition around a lung vessel in a two month-old male SOCS-2$^{-/-}$ mouse. **e**, Lung of age- and sex-matched wild-type mouse.

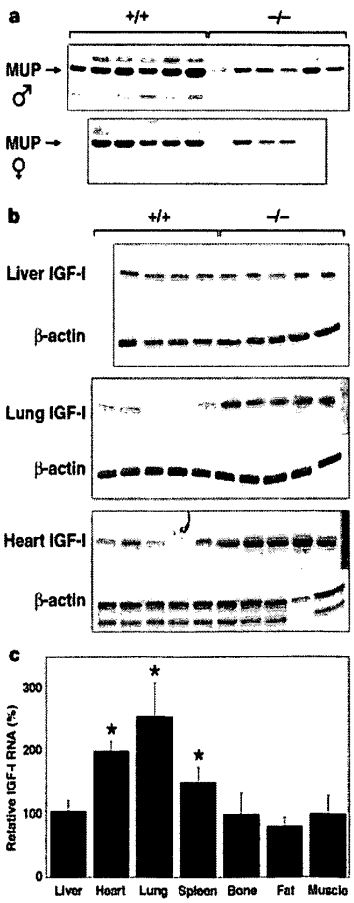

Figure 4 Deregulated growth hormone signalling in SOCS-2$^{-/-}$ mice. **a**, Decreased MUP in urine samples from male and female SOCS-2$^{-/-}$ mice. **b**, RNase protection assays of IGF-I expression in tissues of SOCS-2$^{-/-}$ mice. In **a** and **b**, each lane reperesents a sample from an individual SOCS-2$^{-/-}$ or age- and sex-matched wild-type mouse. **c**, Expression of IGF-I RNA in tissues of male SOCS-2$^{-/-}$ mice, expressed as a percentage of expression in age- and sex-matched wild type mice. Data represents mean ± s.d. for comparison of at least four pairs of mice. Asterisk, $P < 0.05$.

excess growth hormone[8].

These observations indicated that aspects of growth hormone and/or IGF-I signalling might be deregulated in SOCS-2$^{-/-}$ mice. To examine the growth hormone/IGF-I pathway, we first investigated levels of major urinary protein (MUP), a growth hormone pulse-dependent product that is downregulated in transgenic mice over-expressing growth hormone, where the usual pulsatile pattern of growth hormone signalling is disrupted[11]. Consistent with deregulated growth hormone signalling, there was less MUP in samples of urine from each of six male and five female SOCS-2$^{-/-}$ mice than in a similar number of sex-matched wild-type samples (Fig. 4a). Production of IGF-I is also stimulated by growth hormone, and, consistent with deregulated growth hormone action, RNase protection assays revealed increased IGF-I production in several organs including the heart, lungs and spleen of SOCS-2$^{-/-}$ mice (Fig. 4b, c). Excess production was not, however, evident in the liver, bone, fat or muscle. We observed no increase in serum IGF-I concentration in SOCS-2$^{-/-}$ mice (male −/−: 278 ± 74 ng ml^{-1}; +/+: 282 ± 45; female −/−: 310 ± 62; +/+: 298 ± 76; $n = 6$ mice per group), consistent with normal production in the liver, which is the major source of circulating IGF-I (refs 12, 13).

The excess growth in SOCS-2$^{-/-}$ mice reveals a key physiological role for SOCS-2 in the control of postnatal growth by growth hormone/IGF-I. SOCS-2 is induced by growth hormone *in vitro* and *in vivo* and, at least in some tissue culture assays, has a concentration-dependent inhibitory effect on growth hormone signalling[14–16]. Our data provide strong evidence that SOCS-2 is an essential negative regulator of growth hormone signalling *in vivo*, and that its absence leads to increased growth, at least in some organs, through increased local production of IGF-I. The observation that IGF-I production is normal in the livers of SOCS-2$^{-/-}$ mice supports previous studies in which specific deletion of IGF-I in the liver led to low serum concentrations without affecting growth[12,13]. This reinforces the emerging model that autocrine/paracrine actions of IGF-I are of paramount importance. The absence of elevated IGF-I production in some organs of SOCS-2$^{-/-}$ mice may be explained by tissue-specific differences in the induction of SOCS genes by growth hormone[16,17]. As other SOCS genes can be induced by growth hormone and inhibit its actions, at least when overexpressed[15,18,19], other members of the SOCS family may compensate for the loss of SOCS-2 regulation of growth hormone in certain tissues, resulting in normal IGF-I transcription[20]. Nevertheless, as most organs in SOCS-2$^{-/-}$ mice were enlarged, including some in which elevated IGF-I was not detected, our data may indicate that SOCS-2 is also indispensable in regulating IGF-I signalling itself. Indeed, SOCS-2 has been shown to interact with the IGF-I receptor[21,22]. A role for SOCS-2 in regulating both growth hormone and IGF-I signalling in organ-specific contexts is consistent with our observation that SOCS-2$^{-/-}$ mice exhibit characteristics of both growth hormone and IGF-I transgenic mice without entirely recapitulating either phenotype. More detailed analyses of growth hormone and IGF-I signalling in SOCS-2-deficient mice, as well as the precise definition of the temporal and spatial patterns of SOCS-2 expression in response to these cytokines, will further clarify the role of SOCS-2 in growth control.

Methods

Generation of targeted embryonic stem cells and mutant mice

We used polymerase chain reaction (PCR) to generate a genomic SOCS-2 fragment extending ~2.0 kilobases (kb) from the protein initiation ATG. This fragment was fused to the ATG of β-galactosidase using the *Bam*HI site in the plasmid vector pβgalpAloxneo[23]. The 3′ arm, an *Eco*RI fragment extending 3.7 kb downstream from the termination codon, was blunted and ligated into the *Xho*I (blunted) site of pβgalpAloxneo that already contained the 5′ arm. This targeting vector was linearized with *Not*I and electroporated into C57BL/6 embryonic stem cells. Transfected cells were selected in G418 and resistant clones picked and expanded. We identified clones in which the targeting vector had recombined with the endogenous SOCS-2 gene by hybridizing *Eco*RV-digested genomic DNA with a 0.8-kb *Bam*HI–*Nhe*I fragment situated in the 5′ SOCS-2 genomic sequence just outside the targeting vector (Fig. 1). This probe distinguished between the endogenous (16 kb) and targeted (9 kb) SOCS-2 alleles. A targeted embryonic stem cell clone was injected into Balb/c blastocysts to generate chimaeric mice. Male chimaeras were mated with C57BL/6 females to yield SOCS-2 heterozygotes, which were interbred to produce wild-type (SOCS-2$^{+/+}$), heterozygous (SOCS-2$^{+/-}$) and mutant (SOCS-2$^{-/-}$) mice on a pure C57BL/6 genetic background. We determined the genotypes of offspring by Southern blot analysis of genomic DNA extracted from tail biopsies. The deletion of the SOCS-2 coding sequence and subsequent inability to produce SOCS-2 messenger RNA in mutant mice was confirmed in nucleic acid blots performed as described[24]. Northern blots were probed with a full-length SOCS-2 coding region probe and then with a 1.2-kb *Pst*I chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH) fragment.

Haematological and histological analyses

Peripheral blood white cell and platelet counts were determined manually using haemocytometers. Single-cell suspensions from femoral bone marrow and spleen were prepared, and differential counts of peripheral blood, bone marrow and spleen were performed from stained smears and cytocentrifuge preparations. Clonal cultures of 2.5×10^4 adult bone marrow cells were performed in 0.3% agar as described[25]. Cultures were stimulated with recombinant purified GM-CSF, G-CSF, M-CSF, IL-3 (each at 10 ng ml^{-1}), SCF (100 ng ml^{-1}), IL-6 (100 ng ml^{-1}) or Flk-ligand (500 ng ml^{-1}) plus LIF (10^3 U ml^{-1}). Agar cultures were fixed and sequentially stained for acetylcholinesterase, Luxol fast blue and haematoxylin, and the cellular composition of each colony was determined microscopically. Tissue sections were prepared by standard techniques, stained with haematoxylin and eosin, and examined by light microscopy.

MUP analysis

To analyse MUP levels, 6–7-week-old mice were made to urinate before samples were collected 3 and 5.5 h later. Samples were pooled and centrifuged (13,000$g \times 3$ min) before 0.5 μl of supernatant was electorphoresed in 12% SDS-polyacrylamide gels and stained with Coomassie blue.

Growth curves and linear measurements

We weighed cohorts of mice at weekly intervals for 12 weeks from birth. After being killed, animals were pinned down through the oral cavity and lightly stretched by the tail for nose–anus (body length) and anus–tail (tail length) measurements. To measure skeletal dimensions, limbs were subsequently removed and oriented in a consistent manner for X-ray photography and bone length measurement.

IGF-I measurements

We determined IGF-I levels in serum from orbital bleeds using an EIA kit (rat IGF DSL-10-2900, Diagnostic Systems Laboratories) according to the manufacturer's instructions. IGF-I RNA expression was determined in RNase protections assays as described[13] using β-actin as an internal standard.

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Crystal structure of enteropathogenic *Escherichia coli* intimin–receptor complex

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Intimin and its translocated intimin receptor (Tir) are bacterial proteins that mediate adhesion between mammalian cells and attaching and effacing (A/E) pathogens. Enteropathogenic *Escherichia coli* (EPEC) causes significant paediatric morbidity and mortality world-wide[1]. A related A/E pathogen, enterohaemorrhagic *E. coli* (EHEC; O157:H7) is one of the most important food-borne pathogens in North America, Europe and Japan. A unique and essential feature of A/E bacterial pathogens is the formation of actin-rich pedestals beneath the intimately adherent bacteria and localized destruction of the intestinal brush border[2]. The bacterial outer membrane adhesin, intimin[3], is necessary for the production of the A/E lesion and diarrhoea[4]. The A/E bacteria translocate their own receptor for intimin, Tir[5], into the membrane of mammalian cells using the type III secretion system. The translocated Tir triggers additional host signalling events and actin nucleation, which are essential for lesion formation. Here we describe the the crystal structures of an EPEC intimin carboxy-terminal fragment alone and in complex with the EPEC Tir intimin-binding domain, giving insight into the molecular mechanisms of adhesion of A/E pathogens.

Intimin has an amino-terminal bacterial membrane anchor and C-terminal domains needed for Tir binding[6] (Fig. 1). Tir spans the host cell membrane with both its N and C termini in the host cytoplasm and an extracellular intimin-binding domain (IBD)[7]. The crystal structure of the C-terminal EPEC intimin fragment (residues 658–939) at 1.9 Å resolution has three adjacent domains (Fig. 2): immunoglobulin-like (Ig) D1 (residues 658–751) and D2 (residues 752–841), and a C-type lectin-like D3 (residues 842–939). The secondary structural elements and domain boundaries are comparable to the previously determined NMR structure[8], but the overall rod shape of intimin contrasts sharply with the curved NMR architecture (Fig. 2). The observed rod shape of intimin is also maintained in the crystallographic complex with the Tir IBD.

Intimin makes different and minimal crystal packing contacts in the two crystal forms (the complex crystal has a high solvent content of 73%). Thus, the observed elongated shape is unlikely to be a crystallographic artefact. In addition, the crystal structure of intimin matches closely the 2.3 Å resolution crystal structure of the extracellular fragment of *Yersinia pseudotuberculosis* invasin (residues 503–986), a homologue of intimin that binds to β_1 integrins. Invasin has four Ig-like domains (D1–D4) and a C-type lectin-like domain (D5)[9]. Despite a low sequence identity of only 21%, 241 out of the 282 Cα atoms of our intimin structure were matched to invasin with a moderate root mean square (r.m.s.) deviation of 2.9 Å using ALIGN[10]. Intimin D2 and D3 form a superdomain with 1,572 Å^2 of buried surface (calculated using GRASP[11]), much like invasin D4 and D5. Like the Ig-like domain interfaces of invasin, the intimin D1–D2 interface accounts for 450 Å^2 of buried surface. The intimin D1 aligns equally well (~1.3 Å r.m.s. deviation for ~90% matched Cα atoms) with invasin D1, D2 and D3, implying a similar architecture with multiple Ig-like repeats. Furthermore, the N-terminal 539 amino acids of intimin and the N-terminal 489 amino acids of invasin (33% identity) are interchangeable and sufficient to promote bacterial surface localization of the C-terminal fragments[6], implying that both these adhesins have a similar outer

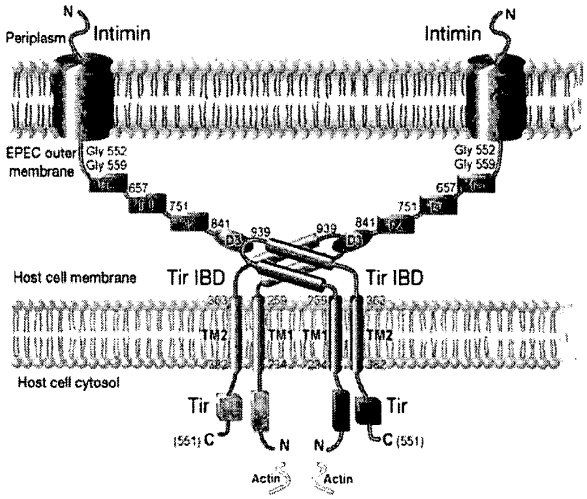

Figure 1 The EPEC/host-cell adhesion interface. The model is based on our structural data of the complex of the C-terminal fragment of intimin (domains D1, D2 and D3) and the extracellular Tir IBD. Intimin is shown in green with domains labelled and boundary residues numbered. The Ig-like domains D0, D1 and D2 are shown as rectangles, and the lectin-like domain D3, which binds to the Tir IBD, as an oval. Tir is shown as a dimer (in pink and dark blue) in the host-cell membrane, and is also labelled and numbered as described for intimin. The Tir IBD is the extracellular component of Tir flanked by the two predicted transmembrane (TM) domains. We observe a dimeric Tir IBD, with the two helices in each monomer forming a four-helix bundle that is stabilized by multiple hydrophobic and hydrogen-bonded interactions. The N-terminal domain of Tir anchors host cytoskeletal components (such as actin) that are needed to form the characteristic A/E lesion on the host-cell surface upon bacterial adhesion.